NATURAL ENVIRONMENT RESEARCH COUNCIL

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Investigating the occurrence and fate of anticancer drugs in sewage treatment works and the wider aquatic environment

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Abstract

The occurrence of pharmaceuticals in wastewater and the wider environment is of growing concern. This thesis focuses on anticancer drugs - a group of biologically-potent and often recalcitrant set of chemicals whose fate and impact on the wider freshwater environment is poorly studied. The aims of this thesis were to prioritise a group of anticancer drugs for environmental monitoring programmes (from the many drugs in use), based on their consumption and fate during wastewater treatment; to undertake a national and regional survey of two commonly used anticancer drugs, cyclophosphamide (CP) and ifosfamide (IF) in wastewater and river water; to assess the performance of a river-based chemical fate model through comparisons with field observations; and to conduct a mass balance for CP in wastewater treatment plants to assess chemical fate during the different stages of wastewater treatment.

Given the large number of anticancer drugs currently in use (>70) a decision support process was developed to ascertain a short list of drugs which are most likely to persist and be released with treated effluent to environmental waters. To do this, accurate consumption data were compiled from a hospital survey in NW England and combined with urinary excretion rates derived from clinical studies. Physical–chemical property data were then compiled along with likely chemical fate and persistence during and after wastewater treatment. A shortlist of 15 chemicals (from 65), including CP and IF, was prioritised based on their consumption, persistency and likelihood of occurrence in surface waters and supported by observational studies where possible. The ecological impact of these 'prioritised' chemicals however is uncertain as the measured concentrations in surface waters generally fall below standard toxicity thresholds, although there is evidence that exposure of aquatic organisms to some of these chemicals may induce low-dose genotoxic effects. This prioritised sub-list of anticancer drugs should prove useful for developing environmental screening programmes and targeted toxicity assays.

To assess the occurrence of anticancer drugs in wasterwaters both CP and IF were measured in raw influent and final effluent waters from fourteen STPs located across England using a sensitive analytical method. CP was detected in both wastewater influent and effluent with mean (SD) concentration of 4.1 ng/L (4.8) and 6.6 ng/L (6.5), respectively, in agreement to measured ranges from a limited number of studies conducted in Europe and elsewhere. IF was only detected in four wastewater samples with the highest concentration being observed in wastewater effluent at 0.77 ng/L (cv = 24.3% (n=3)) and possibly reflecting the relatively lower consumption of this drug relative to CP. Additional monitoring was conducted in the rivers Calder, Darwen and Ribble (North West UK) with CP present at 5 of the 6 river locations with concentrations ranging from 0.41 to 3.71 ng/L. All these rivers receive treated wastewater effluent from sewage treatment works serving different population sizes, with CP measured in river water some ~20 miles downstream of the nearest STP, indicating the widespread dispersal and persistence of this chemical.

CP and IF were measured systematically down the Rivers Aire and Calder in NE England and the results compared to a GIS-based water quality model (LF2000-WQX) used to predict CP and IF distributions in the two rivers, using regional consumption data and subsequent release quantities from STPs. CP was detected in 90

% of river samples, apart from rural/uplands sites located at the source of the River Aire and Calder, respectively. CP presented the highest concentration, ranging from 0.17 to 4.53 ng/L (average 1.14 ng/L). IF was seldom detected in the sampled sites and concentrations ranged from < LOD to 1.82 ng/L (average 0.51 ng/L). Model results showed a fair agreement to the measured data for CP in the River Aire, discrepancies arise as the river progressed further downstream where the modelled data was lower than the measured data. A significant input of CP from Leeds STP at A7 (STP-1) saw the continuing rise in CP despite the increase in river flow. At the lower end of the Calder (pre-confluence with the River Aire) a spike in CP is detected far beyond the modelled value. A risk assessment was carried out to establish the potential adverse effects of anticancer drugs in the river catchment. All calculated risk quotients were below 1, showing no significant risk to aquatic organisms. However, long term toxicity studies for these chemicals are needed to define the environmental stress produced by their continuous exposure and induction.

The fate and removal efficiency of cyclophosphamide (CP) and ifosfamide (IF) were investigated in two conventional sewage treatment plants (STP-S and STP-C) during different stages of waste water treatment. Overall average concentrations of CP were 1.17±1 ng/L in the two plants, which is lower than recent measurements conducted elsewhere. Grab-samples were coordinated with the hydraulic residence time of wastewater in each of the treatment stages in order to monitor changes in CP concentrations in the same parcel of water as it passed through the STP. Interestingly, concentrations of CP were observed to increase from raw influent to final tertiary-treated effluent and this is likely to be attributable to the degradation of a CP-metabolite and subsequent 'liberation' of the parent CP as the metabolite passes through the various sewage treatment processes. This observation, apparent in both studied STPs, has implications for chemical fate modelling of anti-cancer drugs, especially if STP influent loads are used to predict subsequent fluxes to receiving waters rather than final effluent values. Moreover, this increase in concentrations made a mass balance difficult to achieve, but highlighted that elimination/removal of CP in wastewater during primary to tertiary processing is very low (<20%). The calculated fluxes of CP with final effluent discharge were 3.16- 6.48 g/year for STP-S and 4.56 -51.57 g/year for STP-C and highlight that STPs are a continuing source of highly water-soluble, recalcitrant anticancer drugs to the environment.

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List of abbreviations

5-FU - 5-fluorouracil
AML – acute myeloid leukaemia
APCI – atmospheric pressure chemical ionisation
AS – activated sludge
ATC system – anatomical therapeutic chemical classification system
Cmax – maximum serum concentration
CP – cyclophosphamide
d4-CP - deuterated cyclophosphamide
DC – direct current
DNA - deoxyribonucleic acid
EC_{50} - half maximal effective concentration
FS – full scan
GCMS – Gas chromatography mass spectrometry
GP – general practitioner
HESI – heated electrospray ionisation
HPLC – high performance liquid chromatography
HSRM – highly selected reaction monitoring
IF – ifosfamide
K_{oc} – soil organic carbon-water partitioning coefficient
K_{ow} – octanol-water partition coefficient
LC_{50} – Half maximal lethal concentration
LC-MS/MS – liquid chromatography tandem mass spectrometry
LOD – limit of detection
LOELs – low observed effect levels
MDL – method detection limit
MEC – measured environmental concentration
MoA – mode of action
NOELs – no observed effect levels
PEC – predicted environmental concentration
PNEC – predicted no effect concentration
$Q_1q_2Q_3$ - triple quadrupole
RF – radio frequency
RNA - ribonucleic acid
RPC – reverse phase chromatography
RQ – risk quotient
SIM – selected ion monitoring
SPE – solid phase extraction
STP – sewage treatment plant
TIC – total ion monitoring
UV – Ultraviolet
WHO - world health organisations

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I. Prioritising anticancer drugs for environmental monitoring and risk assessment purposes

Victoria Booker, Crispin Halsall, Neville Llewellyn, Andrew Johnson, Richard Williams

The author contributions are as follows: Victoria Booker was responsible for all aspects of data collection, data analysis and writing of the manuscript; Crispin Halsall and Andrew Johnson was responsible for providing feedback on study design/corrections to the manuscript. Neville Llewellyn and Richard Johnson were collectively responsible for initial conception of the research.

II. A survey of two common-use anticancer drugs in WWTPs and receiving waters: chemical fate and river water loads.

Victoria Booker, Crispin Halsall, Neville Llewellyn, Monika Jurgens, Andrew Johnson, Richard Williams and Gloria Pereira

The author contributions are as follows: Victoria Booker was responsible for all aspects of data analysis and writing of the manuscript and some aspects of data collection (NW river catchment); Crispin Halsall and Andrew Johnson was responsible for providing feedback on study design/corrections to the manuscript. Neville Llewellyn was responsible for providing the analytical method used in the analysis and Monika Jurgens was responsible for collection of the composite samples.

III. Tracking the fate of a common anticancer drug during wastewater treatment and release to receiving waters

Victoria Booker, Crispin Halsall, Neville Llewellyn, Monika Jurgens, Andrew Johnson, Richard Williams and Gloria Pereira

The author contributions are as follows: Victoria Booker was responsible for all aspects of data collection, analysis and writing of the manuscript. Crispin Halsall and Andrew Johnson was responsible for providing feedback on study design/corrections to the manuscript. Neville Llewellyn was responsible for providing the analytical method used in the analysis.

IV. Modelling and measurement of the anticancer drugs, cyclophosphamide and ifosfamide, in the River Aire and Calder basin (UK).

Victoria Booker, Crispin Halsall, Neville Llewellyn, Monika Jurgens, Andrew Johnson, Richard Williams and Gloria Pereira

The author contributions are as follows: Victoria Booker was responsible for all aspects of data collection, analysis and writing of the manuscript; Crispin Halsall and Andrew Johnson was responsible for providing feedback on study design/corrections to the manuscript. Neville Llewellyn was responsible for providing the analytical method used in the analysis. Richard Williams was responsible for running the LF2000-WQX model for the selected chemicals.

Introduction

1. Introduction

This thesis investigates the environmental occurrence of a specific group of pharmaceuticals - anticancer drugs –with a growing number of studies showing their occurrence in sewage wastewaters and receiving waters (Yin et al., 2010, Kümmerer et al., 1997, Buerge et al., 2006, Garcia-Ac et al., 2009, Martín et al., 2011, Coetsier et al., 2009, Zuccato et al., 2000, Valcárcel et al., 2011). Over the last 15 years there has been a rise in the number of scientific papers examining the occurrence of pharmaceuticals in waste water systems, with concern about the impact of these chemicals on the aquatic environment and risk to human health through consumption with drinking water (Johnson et al., 2008, Booker et al., 2014, Besse et al., 2012). Anticancer drugs represent a toxicologically potent group of chemicals given that some of these compounds are designed to be genotoxic and can accelerate cell apoptosis. The potential longevity of these chemicals and their resistance to breakdown during wastewater treatment processes is therefore of concern and warrants research into their environmental occurrence, behaviour and impact. This initial chapter serves as the basis for the significance and rationale of the papers included in the thesis and the work conducted over the past three years. By definition, anticancer drugs are cytotoxic in action are continually released through hospital/municipal wastewaters, and as such have been increasingly reported in the environment as analytical methodologies have been developed or improved (Llewellyn et al., 2011, Buerge et al., 2006, Castiglioni et al., 2005, Moldovan, 2006). Major rivers such as the River Thames in SE England receive a wide array of pharmaceuticals from the large number of STPs that discharge to the river (Rowney et al., 2009). As river water is abstracted for drinking water then risks to human health through exposure to very low doses of these chemicals has to be assessed. At present

pharmaceuticals are not legislated for in wastewater effluents or receiving waters due to the lack of observational studies and knowledge about their fate and impact. These chemicals are not currently included in priority lists of hazardous substances in the EU's Water Framework Directive (EU Water Framework Directive (no date)). Furthermore, negative aspects associated with the risk they pose to the wider environment needs to be balanced with their overriding beneficial use in the treatment of cancers through chemotherapy.

This chapter details the physical and chemical properties of the anticancer drugs investigated, their sources and predicted fate in the environment. Their release and pathways into the environment is described. There are still many uncertainties regarding the ultimate fate of these pollutants in the environment. Their occurrence in drinking water is uncertain due to the limitations of the analytical method for this challenging group of chemicals and lack of observational/monitoring studies. The chapters in this thesis go some way to explaining the behaviour of this class of pharmaceutical in wastewater and river water and the processes affecting their fate.

1.1. Aims and Objectives

The overarching aim of this thesis is to investigate the occurrence of anticancer drugs in the aquatic environment in order to verify their sources and quantify their release from sewage treatment plants and occurrence in receiving waters. This will be achieved by the following objectives:

1. To prioritise commonly used anticancer drugs for environmental screening purposes by critically reviewing their consumption, metabolism rates, physical-chemical properties and degradation pathways in the environment.

2. Conduct a nation-wide survey of two commonly used anticancer drugs in sewage treatment plant influent and effluent wastewaters to acquire measured environmental concentrations (MEC).
3. Conduct a mass balance tracking the fate of a commonly used anticancer drug in sewage treatment plants to assess the chemical behaviour and loss between key wastewater treatment processes.
4. To investigate anticancer drugs in a well characterised river basin in the North East (NE) England to determine concentrations in river water and test a catchment 'low-flow' chemical fate model for anticancer drugs.

1.2. Thesis structure

This thesis is based around four key manuscripts that represent the core of the research work and address the objectives listed above. Chapter 2 introduces widespread occurrence of pharmaceuticals and personal care products in the environment utilizing the literature to present the relevant historical and scientific context necessary to understand the context to the research covered in this thesis. Chapter 3 discusses the current knowledge on the two key anticancer drugs that were the focus for the field and laboratory work conducted in this thesis. The analytical methods, including laboratory protocols and instrumentation, as well as the challenges arising from these methods, are discussed in chapter 4. The scientific papers that comprise the core of this thesis are located in chapter 5, followed by the conclusions and recommendations for further research in chapter 6. The papers included in chapter 5 are:

I. Prioritisation of anticancer drugs consumed within the North West (NW) England from a detailed hospital survey. Priority list finalised by

evaluating the PECs of each anticancer drug with regards to their physical chemical profile and likely environmental fate.

II. Nationwide survey of cyclophosphamide and ifosfamide in fourteen sewage treatment plants operating different types of treatment, within a regional river basin study in the NW England.

III. Mass balance of cyclophosphamide and ifosfamide conducted in two sewage treatment plants in NW England to investigate the reactivation of cyclophosphamide.

IV. A detailed river basin study in NE England with MECs and PECs for cyclophosphamide and ifosfamide.

2. Pharmaceuticals and personal care products

2.1. Definitions and classifications

A pharmaceutical is defined by EU law as 'any substance or combination of substances presented as having properties for treating or preventing disease in human beings' (European commission enterprise (no date)). Medical advances have led to an increasing demand and rising number of agents available in pharmacotherapy. The Anatomical Therapeutic Chemical Classification System (ATC system) is a widely used classification system for pharmaceutical agents, categorised by mode of action in a five tiered system. The first two tiers of the ATC are shown in Table 2.1, demonstrating the widespread application and number of different agents obtainable in hospitals, general practitioners (GPs), pharmacies and supermarkets.

Table 2.1: A list of pharmaceutical classes and their applications according to the ATC system, tiers 1 and 2

Code	Content	
A	Alimentary tract and metabolism	A01-stomatological, A02-acid related disorders, A03-gastrointestinal, A04-antiemetics and antinauseants, A05-bile and liver therapy, A06-constipation, A07-antidiarrheals, intestinal anti-inflammatory agents, A08-antiobesity, A09-digestives, A10-diabetes, A11-vitamins, A12-minerals, A13-tonics, A14-anabolic agents, A15-appetite stimulants, A16-other
B	Blood and blood forming organs	B01-antithrombotic agents, B02-antihemorrhagics, B03-antianemic, B05-blood substitutes, B06-other hematological agents
C	Cardiovascular system	C01-cardiac therapy, C02-antihypertensives, C03-diuretics, C04-peripheral vasodilators, C05-vasoprotectives, C07-beta blocking agents, C08-calcium channel blockers, C09-renin-angiotensin system, C10-lipid modifying agents
D	Dermatologicals	D01-antifungals, D02-Emollients, D03-treatment of wounds and ulcers, D04-antipruritics, D05-antipsoriatics, D06-antibiotics and chemotherapeutics, D07-corticosteroids, D08-antiseptics and disinfectants, D09-medicated

		dressings, D10-anti-acne, D11-other
G	Genito-urinary system and sex hormones	G01-gynecological antiinfectives and antiseptics, G02-other gynecologicals, G03-Sex hormones, G04-urologicals
H	Systemic hormonal preparations, excluding hormones and insulins	H01-pituitary and hypothalamic hormones, H02-corticosteroids, H03-thyroid, H04-pancreatic, H05-calcium homeostasis
J	Anti-infective for systemic use	J01-antibacterials, J02-antimycotics, J04-antimycobacterials, J05-antivirals, J06-immune sera and immunoglobulin's, J07-vaccines
L	Antineoplastic and immunomodulating agents	L01-antineoplastic agents, L02-endocrine therapy, L03-immunostimulants, L04-immunosuppressants
M	Musculo-skeletal system	M01-anti-inflammatory, M02-topical products, M03-muscle relaxants, M04-antigout, M05-drugs for bone disease, M09-other
N	Nervous system	N01-anesthetics, N02-analgesics, N03-antiepileptics, N04-anti-parkinson drugs, N05-psycholeptics, N06-psychoanaleptics, N07-other
P	Antiparasitic products, insecticides and repellents	P01-antiprotozoals, P02-anthelmintics, P03-ectoparasiticides
R	Respiratory system	R01-nasel preparations, R02-throat preparations, R03-obstructive airway diseases, R05-cough and cold, R06-antihistamines, R07-other
S	Sensory organs	S01-ophthalmologicals, S02-otologicals, S03-Ophthalmological and otological preparations
V	Various	V01-allergens, V03-other therapeutic, V04-diagnostic agents, V06-general nutrients, V07-other non-therapeutic, V08-contrast media, V09-diagnostic radiopharmaceuticals, V10-therapeutic radiopharmaceuticals, V20-surgical dressings

2.2. History and usage

Historically, pharmaceuticals were distributed by small scale apothecaries in the 17th century that discovered drugs through identifying the active ingredient from traditional remedies or by unexpected discovery. In the 19th century, apothecaries started wholesale production of drugs such as morphine and quinine. In the late 19th century, dye and chemical companies started pharmaceutical production through medical applications found in their products.

The next progression in pharmaceutical growth was in the earlier 20th century, when Paul Ehrlich focused research by postulating that agents (i.e. dyes) would react with disease causing organisms (Ehrlich, 1877). Breakthroughs in the development of synthetic vitamins, sulphonamides, antibiotics, hormones, psychotropics, antihistamines and new vaccines progressed from 1930 to 1960, during this period research focus changed to synthetic chemistry (Dowling, 1972, Tobbell, 2008). It was the discovery of antimicrobials that gave rise to the high profits in pharmaceutical companies, resulting in their expansion. Safety regulations were introduced in 1938 in the U.S. after sulfanilamide (an antibiotic) was developed from diethylene glycol and caused death to more than 100 people by ingestion of the elixir. The drug was almost wholly excreted in the urine, however for patients with poor renal function a diminished ability to excrete sulfanilamide resulted in an accumulation of this chemical in the body (Kanthak and Pickering). In England, in 1956, a revision of the Therapeutic Substances Act was ruled to bring more substances under government control and set formal standards for the testing and manufacturing of pharmaceuticals. During the past two decades, the pharmaceutical industry has brought a new wave of medicines to market, such as drugs for viral and retroviral infection and drugs to cure or delay the onslaught of cancer. This had led to the rise of pharmaceuticals with between 50,000 and 100,000 being commercially manufactured by industry (2006).

2.3. Administration

The administration of a pharmaceuticals to a patient include intravenous, oral or by other means of administration such as, intranasal, topical, inhalation and rectal.

2.4. Environmental occurrence and risk assessments

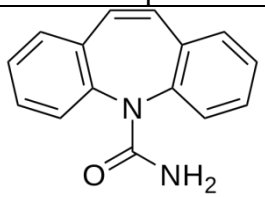
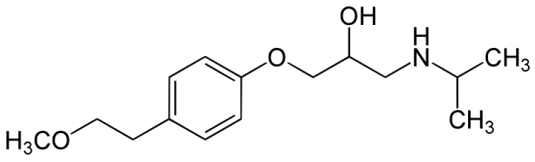
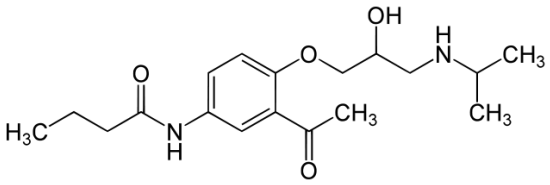
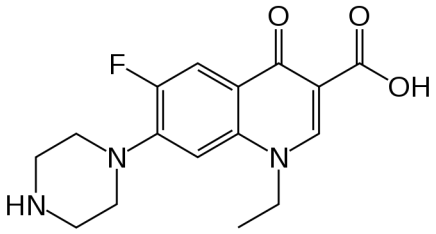
Pharmaceuticals are often viewed as ‘emerging contaminants’ in the aquatic environment, where they are released in some water bodies and display a subsequent

threat to aquatic ecosystems. Pharmaceuticals indirectly enter the environment via wastewaters (i.e. hospital, domestic and industrial) through the excretion of the non-metabolised drug following administration to patients'. The pollution to waterways and soils is not only associated with STPs, veterinary medicines enter the environment primarily through runoff from manure treated farmlands (Kim et al., 2008). The water framework directive sets out strategies against the pollution of water by prioritising a list of pharmaceuticals that present a significant risk to the aquatic environment. The sewage treatment plants (STPs) receiving the wastewaters are not designed to remove these micropollutants and since some compounds are not fully eliminated, the STPs act as a carrier for their release into the environment e.g. carbamazepine (Clara et al., 2004, Ternes, 1998, Vieno et al., 2007).

Within STPs pharmaceuticals are primarily eliminated by biodegradation and sorption (Castiglioni et al., 2006); however the main purpose of the STPs is to eliminate dissolved organic matter, nutrients and solids (Vieno et al., 2007). Given that many of the pharmaceuticals are hydrophilic (i.e. relatively low K_{ow} and K_{oc} values) their sorption to sludge is often limited. Many other factors affect the rate of elimination during sewage treatment e.g. treatment process type, dilution of raw sewage, temperature, solids retention time and hydraulic retention time. Elimination rates can vary from plant to plant and in the same plant at different time periods (Castiglioni et al., 2006). Investigations have demonstrated that a change in operating conditions (e.g. sludge retention time) can effectively improve the elimination of some pharmaceuticals (Cirja et al., 2008, Clara et al., 2005, Andreozzi et al., 2003). Therefore, comparison of operational/control strategies in STPs is a promising tool to test the relative removal effectiveness of these compounds.

Carbamazepine is well documented and a very persistent antiepileptic drug in the environment, making it a good 'model' pharmaceutical for anthropogenic influences in the environment. Carbamazepine removal is negligible and is reported by a number of studies at higher concentrations in the treated sewage rather than in the raw influent (from 20% upto twice as high) (Jones et al., 2005, Clara et al., 2004). The most probable explanation for this is the conversion of carbamazepine glucuronide and other conjugated metabolites back into the parent compound by enzymatic processes (Bahlmann et al., 2014). Cleavage of the glucuronic acid moiety is feasible in STPs as activated sludge (AS) has been found to have glucuronidase activity (Ternes et al., 1999). The presence of a carbamazepine glucuronide was detected at higher concentrations in influent waters and negligible detection in effluent wastewaters supporting this pathway (Vieno et al., 2007). However, this might not be the case for all pharmaceuticals. Elimination efficiencies range from > 80% i.e. norfloxacin, to moderate elimination (40-80%) i.e. acebutolol to poor elimination <40% i.e. metropolol and show no elimination i.e. carbamazepine (Vieno et al., 2007). Table 2.4.1 shows the structure of these four example drugs and their physical chemical properties. The Table illustrates the diverse range of pharmaceuticals that are released into in our sewage wastewaters and receiving waters.

Table. 2.4.1 Important Pharmaceuticals representing different ATC classes along with key physical chemical properties with their removal efficiencies in STPs.

Carbamazepine	Metropolol
 MW (g/mol) = 236.3 Water solubility (mg/L) = 17.7 Log K_{ow} = 2.45 Removal efficiency = -121% (Vieno et al., 2007)	 MW (g/mol) = 267.4 Water solubility (mg/L) = 4780 Log K_{ow} = 1.69 Removal efficiency = 17% (Vieno et al., 2007)
Acebutolol	**Norfloxacin**
 MW (g/mol) = 336.4 Water solubility (mg/L) = 259 Log K_{ow} = 1.71 Removal efficiency = 47% (Vieno et al., 2007)	 MW (g/mol) = 319.3 Water solubility (mg/L) = 178000 Log K_{ow} = -1.03 Removal efficiency = nd (not detected) (Vieno et al., 2007)

The literature offers a wide range of studies with extensive measuring campaigns analysing the occurrence of pharmaceuticals in STP influent and effluent wastewaters. Table 2.4.2 shows some highly consumed pharmaceuticals and a corresponding study reporting their occurrence in wastewaters.

Table 2.4.2. STP influent and effluent concentrations of highly consumed pharmaceuticals

Pharmaceutical	Type of Pharmaceutical	ATC	Influent concentration (ng/L)	Effluent concentration (ng/L)	Ref
Ibuprofen	Nonsteroidal anti-inflammatory drug	M01AE01	1320-6840	1320-6840	(Weigel et al., 2004)
Diazepam	Psychoactive agent	N05BA01	<LOD - 320	<LOD - 126	(Bones et al., 2007)
Carbamazepine	Anticonvulsant	N03AF01	880 - 4026	69 -3581	(Huerta-Fontela et al., 2008)
Atorvastatin	Statin (lower blood cholesterol)	N03AF01	<LOD – 280	<LOD	(Vanderford and Snyder, 2006)

Only human exposure is monitored during drug development and this scrutiny is not always extended to the negative impact that they can cause in the environment. In many cases, these pollutants can pose a significant risk on the environment and human health (Ternes et al., 2004). There is a surplus of literature addressing the potential adverse effects of pharmaceuticals in the environment, but only a handful of studies report effects such as changes to hormone synthesis, reproduction and mitochondrial oxidation metabolism (Pagano et al., 2001, Nash et al., 2004, Mimeault et al., 2005). Several studies report adverse affects in fish in estrogenic effluents when comparing populations of upstream and downstream wild fish, where results show that fish living downstream from a STP discharge exhibit severe signs of endocrine disruption notable as a higher proportion of intersexed fish (Vasquez et al., 2014, Williams et al., 2003).

With over 50,000 pharmaceuticals commonly used today, risk assessments have been made to help predict the chemicals that are likely to be hazardous to the environment

before adverse effects are seen on the aquatic ecosystem. Currently the most common method for assessing the environmental risk of pharmaceuticals is by deriving a predicted no effect concentration (PNEC) from available toxicity data. The PNEC can be compared to a predicted environmental concentration (PEC) based on usage projections. This strategy is required in both the US and EU to assess the environmental risk of a prospective pharmaceutical. However, most of the drugs detected in environmental compartments were approved before environmental toxicity testing paradigms were established. Rigorous pharmaceuticals testing during their preclinical and clinical development allows determination of dose-response for beneficial and adverse effects demonstrated in their No-Observed-Effect-Levels (NOELs) and Low-Observed-Effect-Levels (LOELs).

Prioritising pharmaceuticals for environmental monitoring and/or risk assessment purposes was identified as a major research need (Boxall et al., 2012) where many publications prioritise by categorising consumption, therapeutic class, mode of action (MoA) or physical-chemical properties (i.e. lipophilicity) (Besse et al., 2012, Caldwell et al., 2014, Berninger and Brooks, 2010, Roos et al., 2012, Kostich and Lazorchak, 2008, Dong et al., 2013). After review of prioritisation methods, Caldwell et al 2014, proposed an approach with 3 steps to identify and prioritise substances for prospective and retrospective risk assessments. Step 1 makes use of mammalian pharmacological data i.e. the maximum plasma concentration after drug administration (Cmax). Step 2 involves consideration of the lipophilicity across environmentally relevant pH ranges and ionization potential and thus bioavailability of a specific pharmaceutical. Such a consideration is critical because over 70% of therapeutics are ionizable at environmental pHs (Caldwell et al., 2014). The final step is to use exposure modelling software to further refine the priority list by considering

the quantity of the pharmaceutical sold and induced into the environment by adjusting the metabolised and removed fractions from STPs (Caldwell et al., 2014).

Are pharmaceuticals an emerging environmental problem or is it just the analytical advances that make them detectable now, when in the past they were present but invisible (Taylor and Senac, 2014). Pharmaceuticals are diverse in structure and don't have similar chemical, physical, structural or biological similarities, the common factor that unites them as a group is use (Taylor and Senac, 2014). They are designed to be biologically active, and therefore considered more harmful. Some pharmaceutical sub-groups may be more toxic than others such as the antineoplastic and immunomodulating agents (ATC class L01). The chemicals in L01 largely target DNA and are cytotoxic in action causing subtle genetic alterations that have the potential to cause long term adverse effects in the aquatic environment. There is a need to perform a risk assessment on the sub-groups of pharmaceuticals to determine, which if any are detrimental to the environment. 'Down the drain' chemicals don't exist solely in the aquatic environment and are present as a mixture of many diverse pharmaceuticals which may act additively enhancing their negative effects.

3. Anticancer drugs

Cancer is a major public health concern with significant mortality, in 2011 over 338,000 newly diagnosed cancers were registered in the UK (Table 3) and 14.1 million in the world were diagnosed in 2012. There is high associated mortality with cancer, with approximately 8.2 million deaths in 2012 worldwide. There are more than 100 different types of cancer, the four major cancers accounted for 54% of the diagnosed cancers in 2011 (UK), breast cancer (15%), lung cancer (13%), prostate cancer (13%) and bowel cancer (13%). Chemotherapy is administered in a standardised regimen, where one or more chemotherapeutic agents are dispensed with the intent to prolong life, reduce symptoms (palliative chemotherapy) and/or provide a curative response in the patient. Chemotherapy is one of the major categories of medical oncology, as is often applied in combination with radiation, surgery and/or hyperthermia therapy. Some chemotherapeutic agents are used outside the ATC L01 classification and used in the treatment of other conditions, including multiple sclerosis and Crohn's disease. In 1941, a patient diagnosed Hodgkin's lymphoma was treated with an experimental drug found to be a hematopoiesis (blood production) suppressor after its use as a chemical warfare agent during WWI (Joensuu, 2008, Krumbhaar, 1919). The first chemotherapy drug to be developed from this research was mustine.

Table 3. UK cancers diagnosed in 2011 and their mortality in 2012. Statistics obtained from http://www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/ (Date assessed 5/03/2015)

Type	No. of diagnosed cancers in 2011 (UK) that contribute to more than 1% of the cancer burden	No. of cancer mortality in 2012 (UK)
Breast	49936	11643
Lung	43463	35371
Prostate	41736	10837
Bowel	41581	16187
Skin	13348	2148
Non-Hodgkin lymphoma	12783	4676
Bladder	10399	5242
Kidney	10144	4252
Cancer of unknown primary	9762	10625
Brain tumours	9365	5187
Pancreatic	8773	8662
Leukaemia	8616	4807
Uterine	8475	2025
Oesophageal	8332	7701
Ovarian	7116	4271
Stomach	7089	4758
Oral	6767	2119
Myeloma	4792	2742
Liver	4348	4514
Soft tissue sarcoma	3272	
Chronic lymphocytic leukaemia	3233	
Mesothelioma	2570	2429
Laryngeal	2360	784
Testicular	2207	63
Other	17806	3037
Total	**338273**	**154080**

3.1. How cancer arises

All cancers arise from genetic mutations that control cell growth and behaviour to allow the cell to be invasive and metastatic. Cancerous cells and normal cells undergo very similar cell division, but in many cases cancerous cells exhibit loss of control of the cell cycle and deregulation leads to tumour formation. Abnormalities in a cancer

cell cycle include rapid cell division, failure to arrest the cell cycle at checkpoints and failure to trigger programmed cell death (apoptosis) in the presence of damaged DNA (CHAFFEY, 2003). Both environmental and genetic factors lead to the accumulation of genetic mutations in oncogenes (genes that promote cancer) i.e. cell cycle inhibitors (p53) (Bian et al., 0000). Figure 3 shows the normal somatic cell cycle which consist of two alternative phases; S phase (DNA is replicated) and M phase (Mitosis where cell division produces two daughter cells). A checkpoint in the middle of mitosis ensures the cell is ready to divide. Gap 1 is where the cells increase in size and go through a checkpoint prior to DNA synthesis. Gap 2 is between DNA synthesis and mitosis, the cells continue to grow and go through another checkpoint control to ensure that everything is ready to enter mitosis.

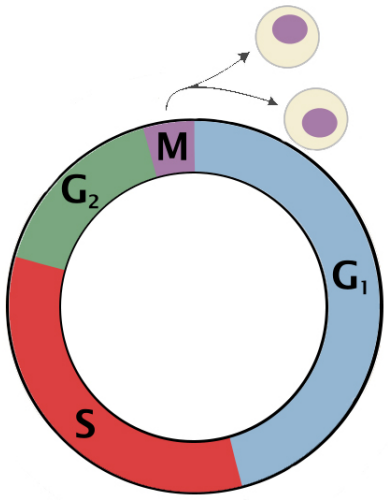


Figure 3.1 Schematic of normal cell cycle control; M (mitosis), G1 (gap 1), S (synthesis), G2 (gap 2)

3.2. Chemotherapy and types of anticancer drug

The majority of chemotherapy drugs act by targeting the cellular processes of rapidly dividing cells and impairing mitosis. Mitosis is prevented by a number of mechanisms, traditional chemotherapeutic agents (cytotoxic) interact with the DNA

and the cellular components relevant for cell division. Understandably, cancers such as acute myeloid leukemia (AML) with high growth rates are more sensitive to treatment with chemotherapeutic drugs than tumours with slower growth rates, as a higher proportion of cells are undergoing cell division at any time (Corrie, no date). The cytotoxic agents are non-cell cycle specific and also harm healthy cells (bone marrow, digestive tract cells and hair follicles) resulting in the common side effects of chemotherapy (myelosuppression, mucositis and alopecia). Modern anticancer drugs (monoclonal antibodies) are considered to be a 'targeted' chemotherapy and target proteins (essential for growth) that are overexpressed in malignant cells.

3.2.1. Alkylating agents

The cytotoxic alkylating agents are anti-proliferative in action as they covalently bind to DNA, RNA and proteins through their alkyl group (C_nH_{2n+1}) forming a crosslink to halt cell proliferation. The electrophilic alkyl group interacts with the nucleophilic nitrogen atom located on the purine ring of the guanine base in DNA (Figure 3.2.1) (Puyo et al., 2014). The alkylating agents can form intrastrand or interstrand crosslink's, by binding to one or both strands of DNA, respectively. The crosslinking causes DNA strand breaks when the cell undergoes replication, thus inducing apoptosis. The alkylating agents are non-cell specific and act at any point during the cell cycle; therefore the cellular effect is dependent and directly proportional to the drug dose.

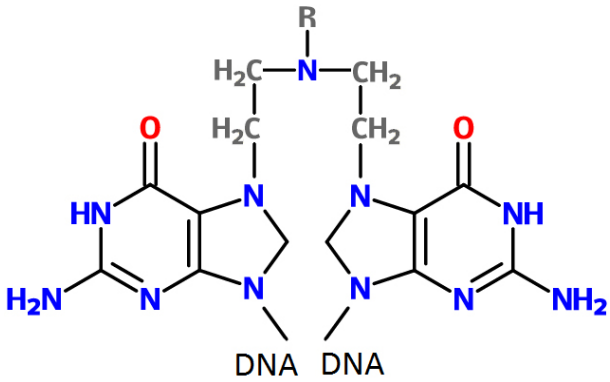
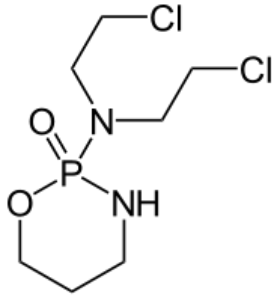
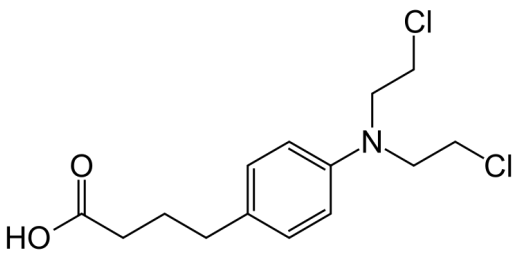
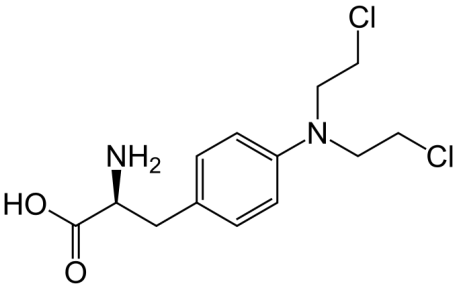
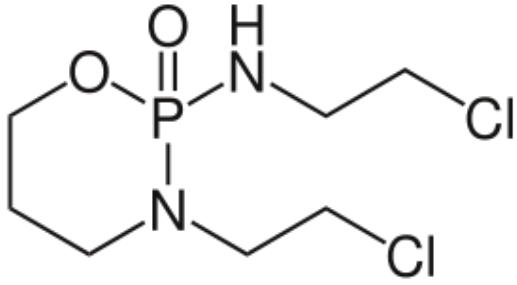
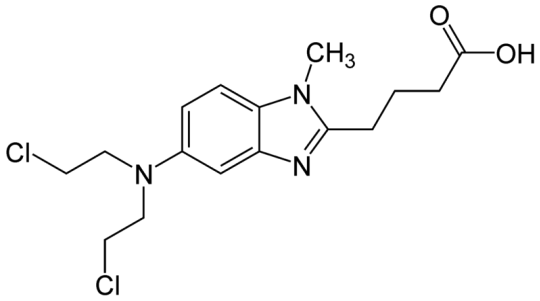
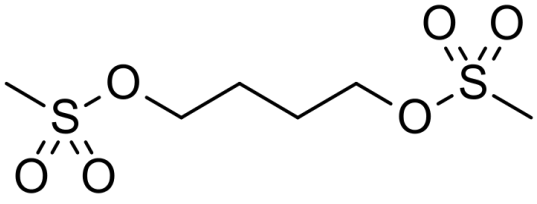
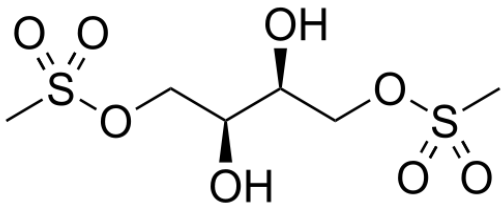
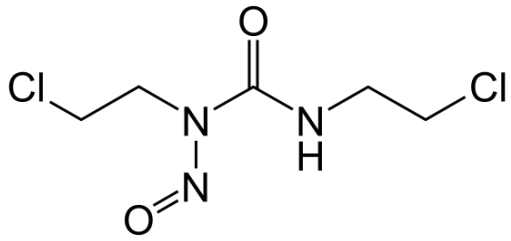
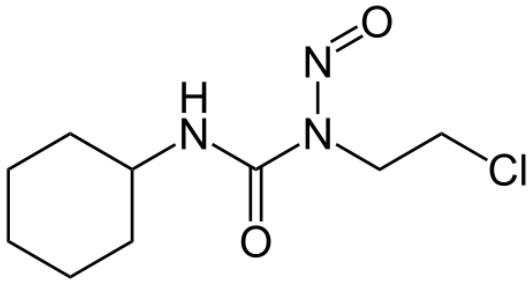
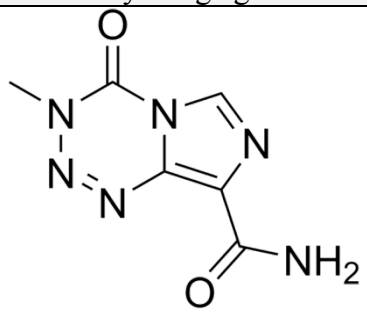
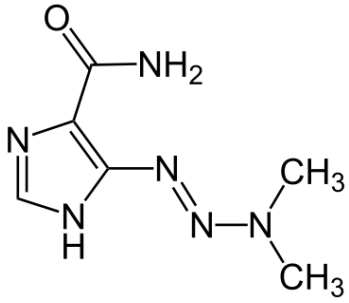


Figure 3.2.1. Crosslinked DNA by alkylating agent

Some of the alkylating agents require activation into their active substances *in vivo*. For example cyclophosphamide an oxazaphosphorine alkylating agent is activated by liver cytochrome P450 (CYP) enzymes into 4-hydroxy cyclophosphamide that has therapeutic activity (Table 3.2.1) (Puyo et al., 2014). Ifosfamide (IF) is an analog of cyclophosphamide and has a similar range of anticancer activity (Weiss, 1991). Both CP and IF have adverse side effects, especially at higher doses, including neurotoxicity, nephrotoxicity (kidney toxicity) and/or bladder toxicity causing an onset of conditions i.e. bladder cancer (Weiss, 1991). Cyclophosphamide is on the world health organisations (WHO) list of essential medicines, where upto 580 medications are listed (other alkylating agents on the WHO list shown by asterisk in Table 3.2.1).

Table 3.2.1. Alkylating agents (asterisk shows medications listed on WHO list of essential medicines)

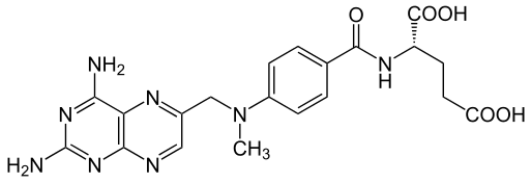
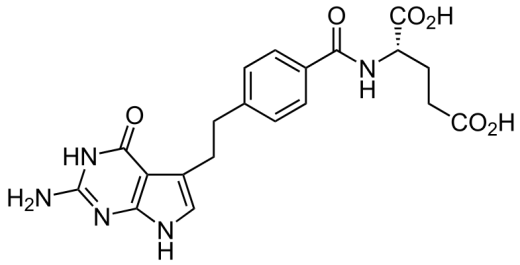
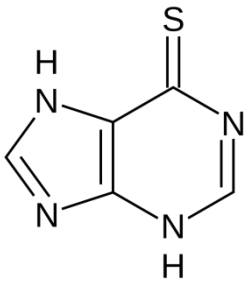
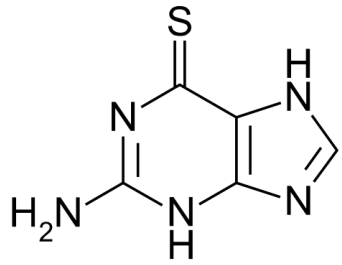
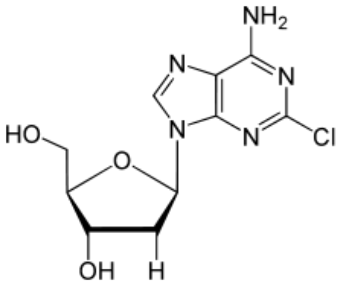
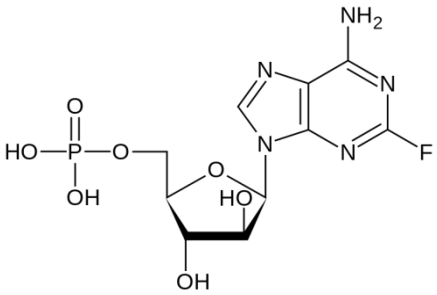
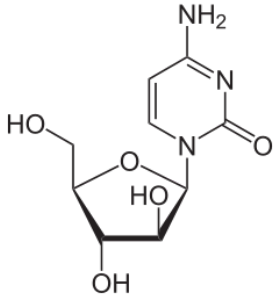
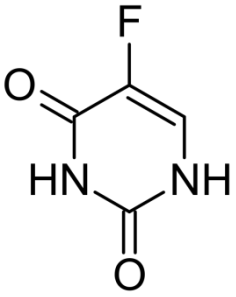
Nitrogen mustard analogues	
 L01AA01 Cyclophosphamide*	 L01AA02 Chlorambucil*
 L01AA03 Melphalan	 L01AA06 Ifosfamide
 L01AA09 Bendamustine	
Alkyl sulfonates	
 L01AB01 Bulsulfan	 L01AB02 Treosulfan
Nitrosoureas	

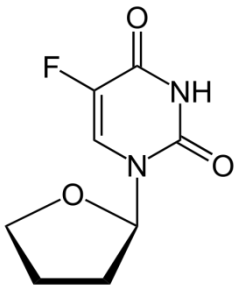
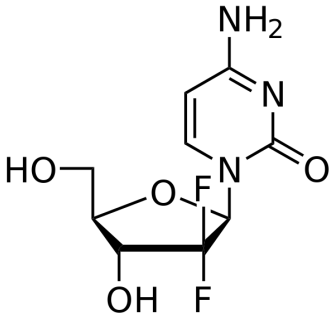
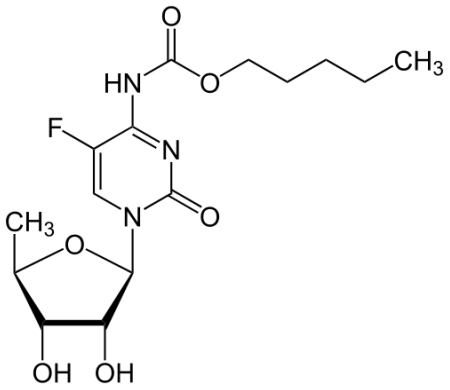
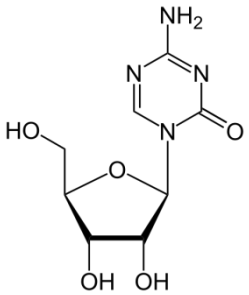
 L01AD01 Carmustine	 L01AD02 Lomustine
Other alkylating agents	
 L01AX03 Temozolomide	 L01AX04 Dacarbazine*

3.2.2. Antimetabolites

Antimetabolites are cell-cycle dependent and act during the S-phase of replication, by impeding DNA and RNA synthesis. Many have similar structures to the nucleotides of DNA and RNA; guanine (G), adenine (A), cytosine (C), thymine (T) and uracil (U) (RNA only). The antimetabolites have well defined MoA and act by interfering with cellular processes required for DNA synthesis (incorporation into DNA/RNA or by inhibiting enzymes) leading to programmed cell death (Peters et al., 2000). Since the antimetabolites only work in the synthesis phase, increasing the dose does not increase their efficiency of treatment.

Table 3.2.2. Antimetabolites (asterisk shows medications listed on WHO list of essential medicines)

Folic acid analogues	
	
L01BA01 Methotrexate*	L01BA04 Pemetrexed
Purine analogues	
	
L01BB02 Mercaptopurine*	L01BB03 Tioguanine*
	
L01BB04 Cladribine	L01BB05 Fludarabine
Pyrimidine analogues	
	
L01BC01 Cytarabine*	L01BC02 Fluorouracil*

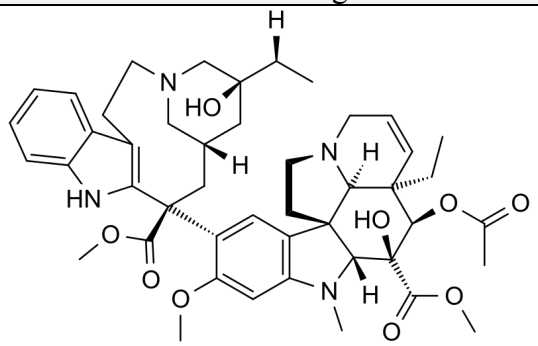
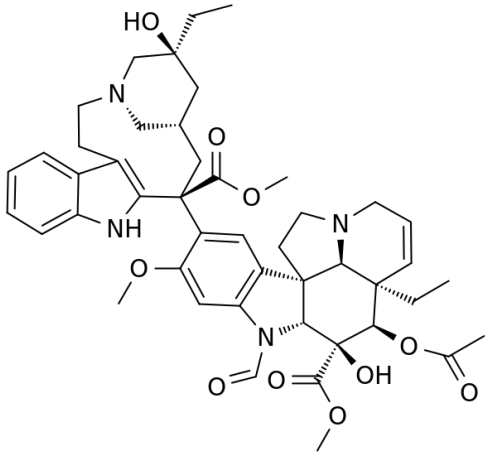
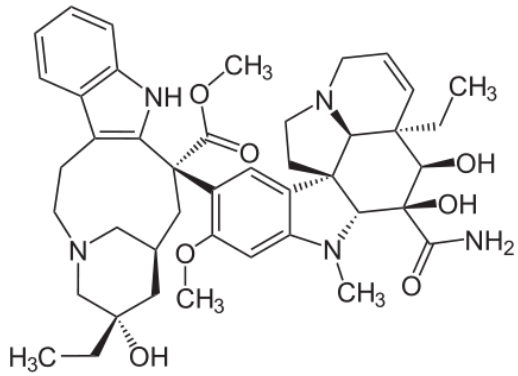
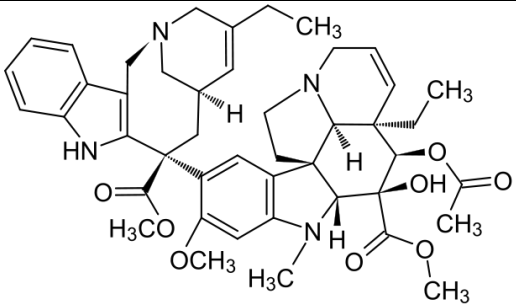
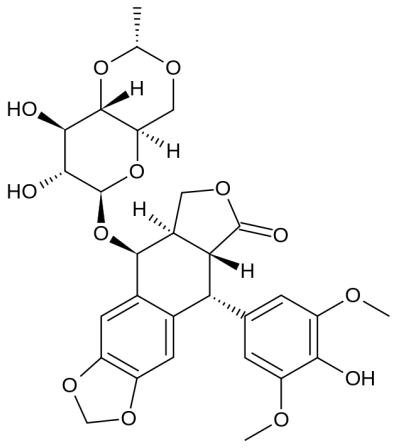
 L01BC03 Tegafur	 L01BC05 Gemcitabine
 L01BC06 Capecitabine	 L01BC07 Azacitidine

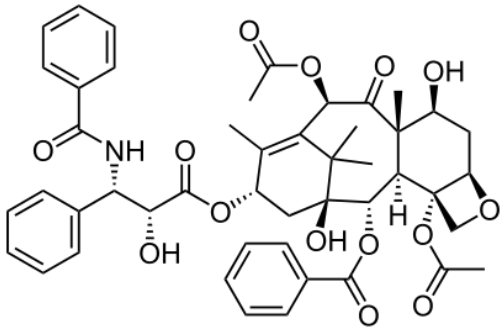
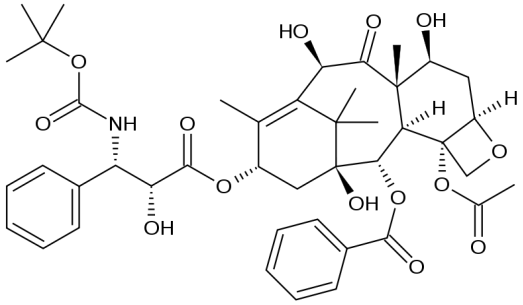
3.2.3. Plank alkaloids and other natural products

The vinca alkaloid chemotherapy agents target a ubiquitous polymer with an essential role in cell division, the microtubules. Tubulin, the building block of microtubules is a globular protein targeted by this class of anticancer drug. Vincristine, vinblastine and vinorelbine (Table 3.2.3) are often used to treat haematological cancers (leukaemia) (Stanton et al., 2011). Where vinblastine inhibits the production of new blood vessels and vincristine destabilises tubulin by reversibly binding to the two sites. The reversible binding makes vincristine particularly powerful as it reattaches to another site, making the assembly of microtubules unsuccessful. Blocking the microtubules is essential to stop the formation of the cytoskeleton, responsible for maintaining the cell structure and providing a platform for intracellular transport and composing the mitotic apparatus essential for cell division. The predominate mode of action of the

vinca alkaloids is preventing the cancer cells from successfully dividing; therefore they are cytotoxic in action (Stanton et al., 2011).

Table 3.2.3. Plant alkaloids and other natural products (asterisk shows medications listed on WHO list of essential medicines)

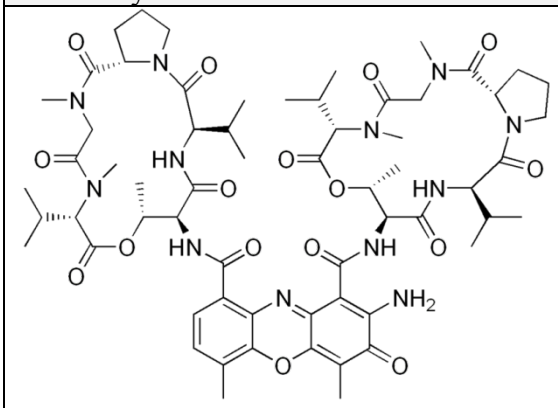
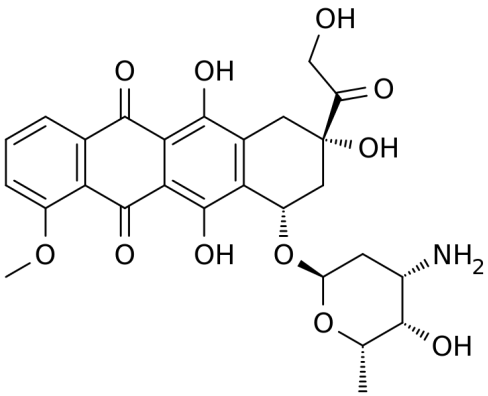
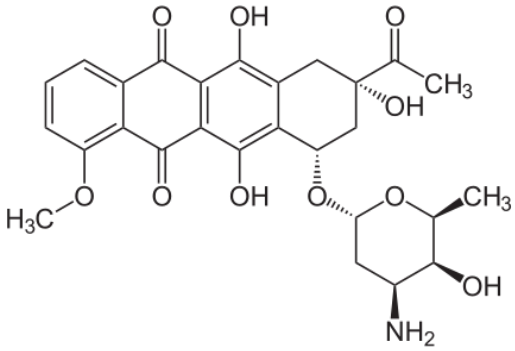
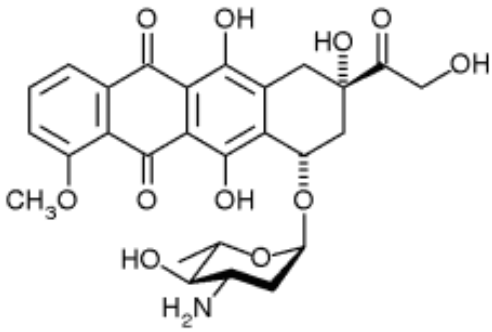
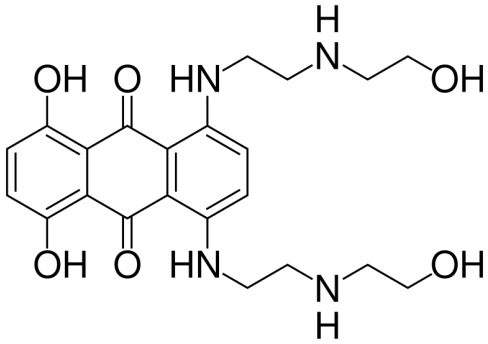
<table>
<tr><td colspan="2">Vinca alkaloids and analogues</td></tr>
<tr><td> 
L01CA01 Vinblastine*</td><td> 
L01CA02 Vincristine*</td></tr>
<tr><td> 
L01CA03 Vindestine</td><td> 
L01CA04 Vinorelbine</td></tr>
<tr><td colspan="2">Podophyllotoxin derivatives</td></tr>
<tr><td>  </td><td></td></tr>
</table>

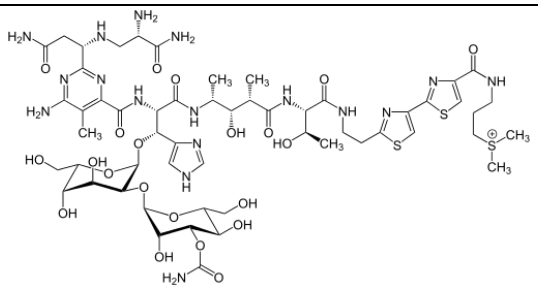
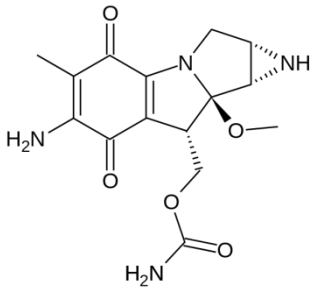
L01CB01 Etoposide*	
Taxanes	
 L01CD01 Paclitaxel*	 L01CD02 Docetaxel*

3.2.4. Cytotoxic antibiotics and related substances

The anthracycline antibiotics are non cell cycle specific and therefore don't require cells to be in a particular phase before their administration. They are used to treat many different cancers and are among the most effective chemotherapy agents developed but, cardiotoxicity as an adverse side effects limits their use (Minotti et al., 2004, Weiss, 1992). Doxorubicin and daunorubicin were first isolated in the mid 20th century from *Streptomyces peucetius* with only small structural differences between them (Table 3.2.4), yet their usage in the chemotherapy regimens varies vividly. The antibiotic anticancer agents employ many different complex MoAs, by interfering/causing (1) oxidation damage (reducing oxygen to reactive oxygen radicals that cause damage to the cell membrane), (2) protein degradation (via proteasome interaction) within the cell and (3) blocking DNA replication by intercalating between DNA and RNA bases leading to apoptosis (Minotti et al., 2004).

Table 3.2.4. Cytotoxic antibiotics (asterisk shows medications listed on WHO list of essential medicines)

Actinomycines	
 L01DA01 Dactinomycin*	
Anthracyclines	
 L01DB01 Doxorubicin*	 L01DB02 Daunorubicin*
 L01DB03 Epirubicin	 L01DB07 Mitoxantrone
Other cytotoxic antibiotics	

	
L01DC01 Bleomycin*	L01DC03 Mitomycin

3.2.5. Other antineoplastic agents

The platinum compounds (Table 3.2.5) are similar in action to the alkylating agents by causing crosslinking of DNA on the N-7 position of guanine, inhibiting DNA repair and/or DNA synthesis (Poklar et al., 1996). Another drug in this category is procarbazine, a methylhydrazine compound. Procarbazine undergoes hepatic metabolism to generate the active species, again it is similar in action to the nitrogen mustard alkylating drugs, except it doesn't contain the fundamental chloroethyl group but instead an N-methyl group (Massoud et al., 2004).

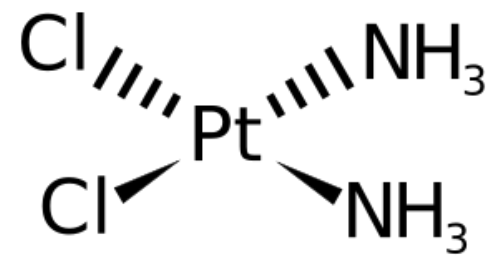
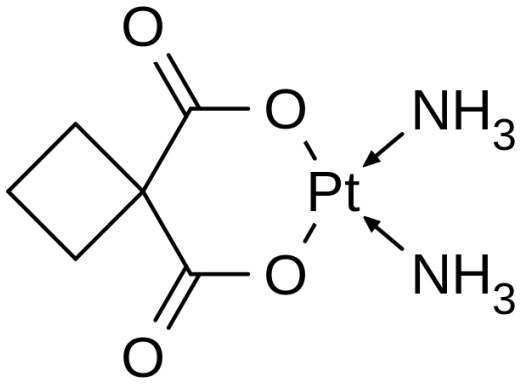
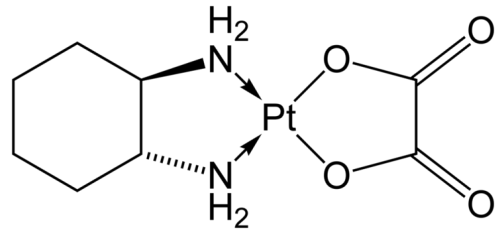
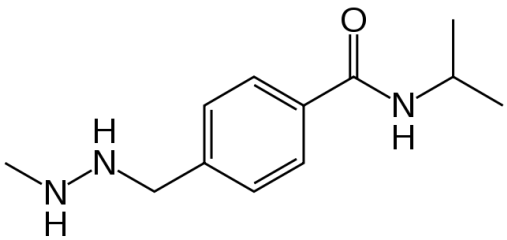
Other drugs in this category are the cancer growth inhibitors; they are a type of targeted biological therapy and include inhibitors such as;

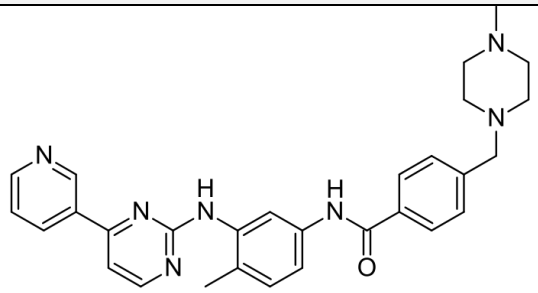
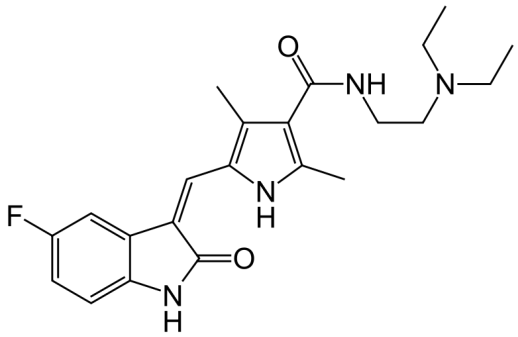
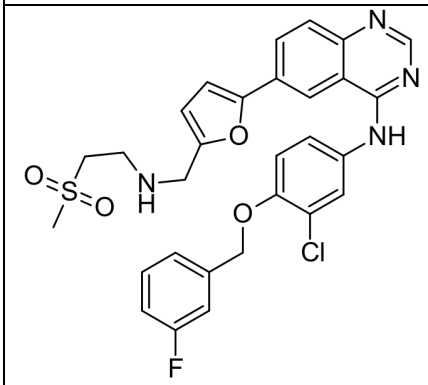
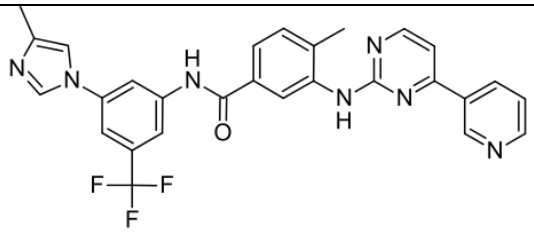
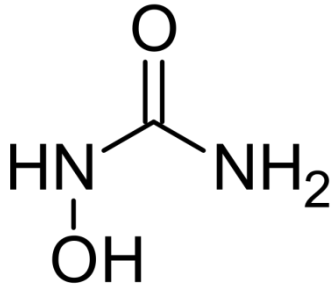
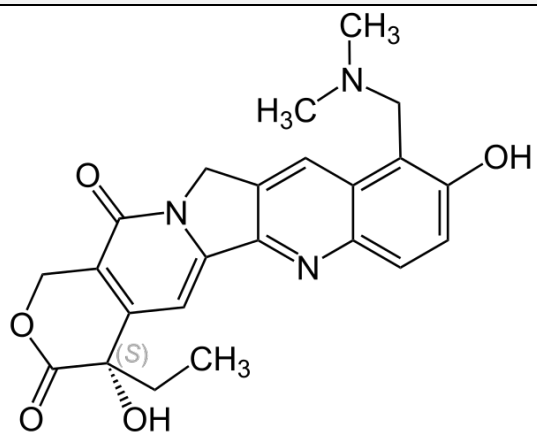
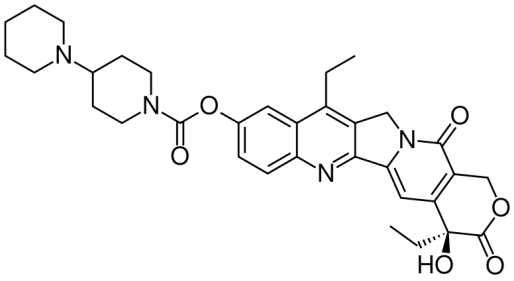
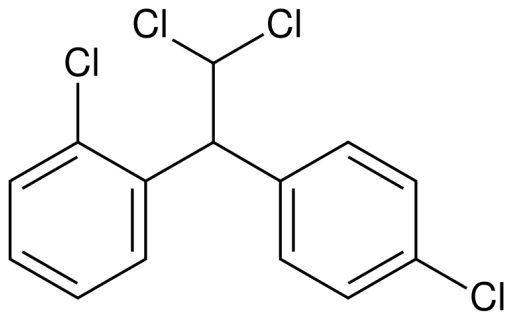
- Tyrosine kinase inhibitors, which block enzymes sending growth signals in the cells (tyrosine kinases)
- Proteasome inhibitors, which block the proteasome enzymes that would normally degrade surplus proteins in the cell, the build-up of surplus proteins induces apoptosis.
- mTOR inhibitors, mTOR is a type of kinase protein that produces cyclins (chemicals that trigger cell growth) and proteins for the development of new blood vessels (an essential for cancerous cells to grow). In some types of

cancer the mTOR is switched on, which makes cancerous cells grow and produce new blood vessels, these inhibitors stop this growth.

- PI3K inhibitors, PI3K act like switches in the cell (turn on proteins such as mTOR – making a cancerous cell grow and multiply). In some cancers P13K is permanently switched on, the hope is that in future treatments blocking the P13K helps stop the uncontrollable growth of cancer cells, at the moment these drugs are in clinical trials.

Table 3.2.5. Other antineoplastic agents (asterisk shows medications listed on WHO list of essential medicines)

Platinum compounds	
 L01XA01 Cisplatin	 L01XA02 Carboplatin*
 L01XA03 Oxaliplatin	
Methylhydrazines	
 L01XB01 Procarbazine*	

Protein kinase inhibitors	
 L01XE01 Imatinib	 L01XE04 Sunitinib
 L01XE07 Lapatinib	 L01XE08 Nilotinib
Other antineoplastic agents	
 L01XX05 Hydroxycarbamide*	 L01XX17 Topotecan
 L01XX19 Irinotecan	 L01XX23 Mitotane

3.3. Anticancer drugs in the environment

Anticancer drugs can enter the environment in the same ways as other pharmaceuticals. For example, after administration, they are metabolized and excreted via urine or faeces. The anticancer drugs have a wide range of metabolism rates and some are excreted in urine in high quantities i.e. over 75% of methotrexate and pemetrexed are excreted as the unchanged parent drug (Blum et al., 2002, Sweeney et al., 2006). Other anticancer drugs such as chlorambucil and capecitabine are readily metabolised and less than 5% are excreted in the urine as the parent drug (Alberts et al., 1979, Straub, 2010). In general the anticancer drugs are highly polar with high aqueous solubility (Booker et al., 2014). They enter sewage treatment plants and are inefficiently removed in STPs, which rely largely on activated sludge mediated through hydrophobic interactions. Biodegradation of anticancer drugs depends largely on the environmental conditions; most anticancer drugs are not readily biodegradable and are considered to be semi-persistent compounds due to their continual release into the environment (Jones et al., 2005). Most anticancer drugs have half-lives of 2 days and environmental persistence times of 8 days (predicted from EPI SUITE). Some STPs have UV systems in place as part of a final treatment; however anticancer drugs such as cyclophosphamide do not have absorption spectra in the UV-range and hence do not undergo direct photolysis. The removal of CP and other anticancer drugs by conventional STP processes is often incomplete and inefficient (Lutterbeck et al., 2015). A select few anticancer drugs have been detected in surface waters at dilute trace levels (low ng/L range). These include cyclophosphamide, ifosfamide, gemcitabine, cytarabine, bleomycin and tamoxifen (Buerge et al., 2006, Zuccato et al., 2000, Valcárcel et al., 2011, Martín et al., 2011, Coetsier et al., 2009).

The anticancer agents are dangerous environmental contaminants that have potent mutagenic, teratogenic, cytotoxic, carcinogenic and/or endocrine disruptor effects in several organisms. They are designed to disrupt and prevent cellular replication by interfering with DNA, RNA and critical pathways, triggering apoptosis of the cancerous cell. After more than 50 years, CP is still a widely used chemotherapy agent worldwide, it has been detected in water bodies and known to be persistent in the aquatic environment. Yet there is still uncertainty over the levels of contamination, persistence and ecotoxicity of this drug in the aquatic environment.

4. Methodology

This chapter presents the analytical methods, laboratory protocols and instrumentation for the analysis of cyclophosphamide and ifosfamide using liquid chromatography tandem mass spectrometry (LC-MS/MS) in the aquatic environment. The first approach in 1996 measured CP and IF in hospital wastewaters by gas chromatography coupled with mass spectrometry (GC-MS) using CP derivatives for internal standardization, aiding a detection limit of 7 ng/L for IF and 6 ng/L for CP (Steger-Hartmann et al., 1996). It is now a common approach to quantify pharmaceuticals with LC-MS/MS using a triple quadrupole ($Q_1q_2Q_3$) because of its specificity and selectivity for the analysis of pharmaceuticals in complex environmental samples (Nussbaumer et al., 2011). For CP and IF $Q_1q_2Q_3$ methods give detection limits of 2ng/L, some methods report limits of detection (LOD) below 1ng/L (Llewellyn et al., 2011, Buerge et al., 2006, Castiglioni et al., 2005, Yin et al., 2010). In these methods, target compounds are extracted either using one or various solid phase extraction (SPE) protocols.

4.1. Tested compounds

Cyclophosphamide ((RS)-N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide) and ifosfamide (N-3-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amide-2-oxide) were analysed in this study. Ethanol was originally used to dissolve CP and IF to ensure the dissolution of the powered standard. All standards were kept in a freezer below -80°C where no degradation occurred. An custom internal standard of deuterated cyclophosphamide (d4-CP) was used to aid quantification.

4.2. Sample collection

Water samples for the majority of studies have been collected in glass amber bottles to avoid photodegradation. In this study the samples were collected in 2.5L amber glass bottles pre-washed and methanol rinsed. On sample collection the bottle was rinsed with the sewage water/receiving water at the sampling point. Samples were kept cool and extracted within 24 hours. All samples were returned to the laboratory where they were stored in the dark at 4°C, filtered (GF/F filters, Whatmann, UK) and spiked with d4-CP within 24 - 48 hours.

4.3. Solid phase extraction

Solid phase extraction is an important technique used in the sample pre-treatment for HPLC, where existing literature indicates SPE as an efficient method of extracting and recovering pharmaceuticals from water samples (Zuccato et al., 2000). SPE is used for six main purposes in sample preparation (1) removal of interferences, (2) concentration of the analyte, (3) desalting, (4) solvent exchange, (5) *In situ* derivatization and (6) sample storage and transport. In this study SPE was used to pre-concentrate and remove interfering compounds. A typical SPE disposable cartridge is depicted in Figure 4.3 with an overview of the processes involved. The application of SPE generally involves four steps (letters denote the steps in Figure 4.3):

a) Conditioning the packing bed: The conditioning step removes any impurities and allows the sorbent to be solvated. Methanol is a commonly used conditioning agent for SPE
b) Sample application: The sample is loaded onto the cartridges in a weak solvent to allow strong retention of the analyte to the cartridge

c) Washing the packing: Removes interferences from cartridge by washing with an intermediate strength solvent that retains the analyte of interest on the cartridge and removes other impurities

d) Recovery of the analyte: Elution of the analyte fraction using a strong elution solvent.

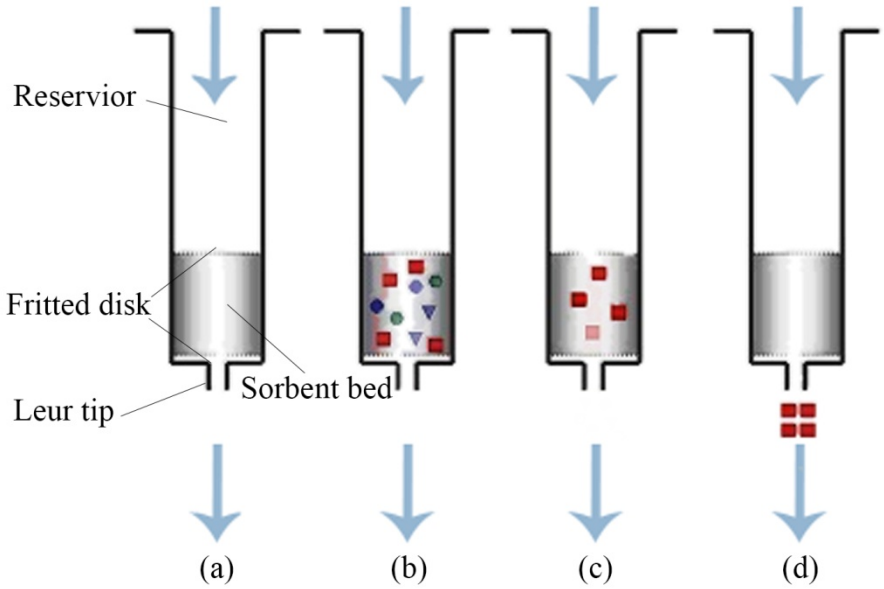


Figure 4.3. SPE cartridge and SPE application (a) conditioning, (b) loading, (c) washing and (d) elution

The basic procedure from primary extraction to preparing the LC-MS/MS sample vial was as follows: Strata X 500mg-6mL SPE cartridges were selected as a sorbent for primary extraction based on their ability to retain polar compounds. The cartridges were loaded onto the autotrace SPE platform and sequentially conditioned with 12 mL ethyl acetate, 12 mL methanol and 12 mL of water at a flow rate of 5 mL/min. The sample lines were pre-cleaned with 25 mL isopropanol and 25 mL of water to prevent sample contamination. The pre-filtered 500 mL effluent samples were then pre-loaded at 6mL/min; an adequate flow rate to allow the pharmaceuticals to be drawn from

their solution (Buerge et al., 2006). Once loaded the sorbent beds of the SPE cartridges were washed with 12 mL HPLC grade water and then rinsed with a solution of HPLC grade water diluted with 40% methanol to remove interfering compounds. The Strata-X cartridges were then dried for 30 minutes by securing a 100 mL cartridge body filled with anhydrous calcium chloride to the inlet of the Strata-X cartridge using SPE column adaptors and drawing air through the stacked cartridges, allowing a solvent change to ethyl acetate for elution. The eluent goes through a second SPE step where the extract is cleaned using Florisil® cartridges in an off-line configuration. The final eluent 10mL 10% methanol dissolved in ethyl acetate (v/v) is collected in a 10 mL round bottom test tube and reduced to dryness using a nitrogen TurboVap® 214 LV (Biotage, Uppsala, Sweden) set to 40 °C and 10 psi. The samples are then dissolved in 500µL mobile phase, vortexed and syringe filtered (0.2 µm, PTFE) into labelled LC vial ready for analysis by HESI-LC-MS/MS (Heated electrospray ionisation).

4.4. Chromatography and identification

It is evident from the literature that a variety of analytical techniques are capable of detecting the anticancer drugs in wastewaters, including high performance liquid chromatography (HPLC) or gas chromatography (GC) coupled with ultraviolet (UV) or mass spectrometry (MS) detection. The anticancer drugs are more commonly analysed by HPLC.

4.4.1. Liquid Chromatography

Chromatography is the physical separation of compounds by their distribution between two phases; a stationary phase and a mobile phase. A mobile phase is described as a liquid, gas or supercritical fluid which percolates through or along the

stationary bed in a definite direction. Figure 4.4.1 shows a schematic of the HPLC system.

The chromatography parameters defined during method development and used in this study are as follows. Solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) were pumped at a predefined flow rate of 300µL/min into a mixing chamber. The sample was then automatically injected using the 10µL partial loop injector mode. Reverse phase chromatography (RPC) was achieved by using a packed µPLC Hypersil GOLD C_{18} column (50 x 2.1 mm 1.9 µm). RPC allows the most polar components of a sample to elute first, increasing the mobile phase polarity and elution time. For the Accela LC, the column oven was set to 50°C and analytes were eluted from the column using the following gradient programme: mobile phase A - 0 min, 95%; 15 min, 0%; 15.5 min, 95%, 20 min, 95%; using these conditions IF eluted at approximately 5.34 minutes and CP at 5.74 minutes. To minimise MS/MS source contamination, the LC flow was only diverted to the MS/MS between three and eight minutes of the acquisition period.

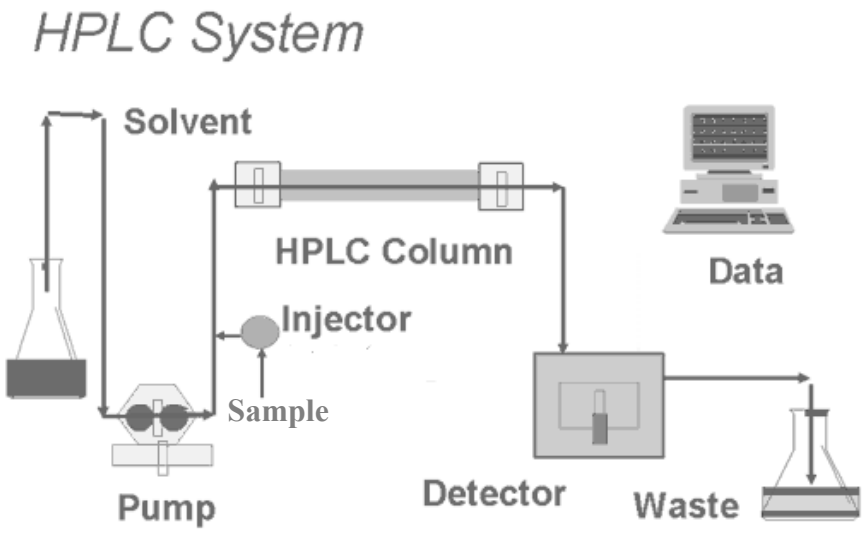


Figure 4.4.1. Schematic of HPLC

4.4.2. Mass spectrometry

A detector must be used to confirm the identity of an analyte. Mass spectrometry stands out in the literature as the most popular method of detection and consists of three main stages; ionization, separation and detection. The MS/MS was operated using a heated electrospray ionisation (HESI) and an atmospheric chemical ionisation (APCI) source. Ionisation firstly involves vaporizing the sample with a nebulizer (nitrogen gas) and bombarding with high energy electrons at a typical energy of 70eV, both the polarity and molecular weight of the compound determine the best source for ionisation. One of the main advantages of ESI is that thermally labile compounds may be ionized without degradation. For CP and IF a single electron is removed from the analyte causing them to become positively ionized and produce a cation (M^+) with a m/z ratio equal to the analytes molecular weight. CP and IF were analysed with both APCI and HESI to see which source obtained the best response.

Once the analyte has been ionized they are separated by their m/z ratios. The separation technique described for this study is the high resolution triple quadrupole. The quadrupole mass filter consists of four parallel 250mm hyperbolic rods with an internal diameter of 6mm where one pair of rods is exposed to a positive direct current (DC) and the other pair is applied with a negative DC. Ions are directed into the quadrupole and oscillate in the electric field. Radio frequency (RF) is applied to both rod pairs but one pair is 180° out of phase. Varying RF and DC voltages systematically alters the trajectory of the ions through the rods and determines which m/z ratios are allowed to reach the detector. Tandem mass spectrometry involves three sets of quadrupole rods in series to enhance ion separation and detection. The first set of rods involves mass separation; the second set of rods is used as a collision

cell to cause fragmentation of the ions transmitted by the first set of rods. The fragmented ions from q2 are channelled into q3 according to their m/z ratios. Data is then collected on the ions structural properties and molecular weight.

The critical part of optimizing MS/MS is choosing a scanning mode suitable for the particular analyte. The two main types of scanning mode are Full Scan (FS) and Selected Ion Monitoring (SIM). The total ion current (TIC) plotted from running in FS traces the ion current and as a compound is eluted from the HPLC column the relative intensity increases and forms a peak, compounds of every mass are plotted in the TIC. Sensitivity for FS will be far less than what is observed in the SIM experiment. SIM monitors over a very small mass range, where the narrower the mass range the more sensitive and only selected compounds are detected and plotted. In this study highly selective reaction monitoring (HSRM) was used, a unique fragment ion produced in q2 is monitored and quantified providing the most sensitivity for both CP and IF. CP and IF tuning was carried out by directly infusing standards (~1mg/L) into the HESI source and MRM transitions, tube lens voltage and collision energies were recorded. The most abundant ions were identified and automatically optimised for both CP and IF. Table 4.4.2 shows the MS ion source parameters.

Table 4.4.2. MS Ion source parameters

Parameter	HESI
Polarity	Positive
Spray voltage (V)	3000
Vaporiser temperature (°C)	350
Sheath gas pressure (arbitrary units)	30
Ion sweep gas pressure (arbitrary units)	0
Auxiliary gas pressure (arbitrary units)	30
Ion transfer capillary temperature (°C)	300
Collision gas pressure (mTorr)	1
Skimmer offset voltage (V)	-5

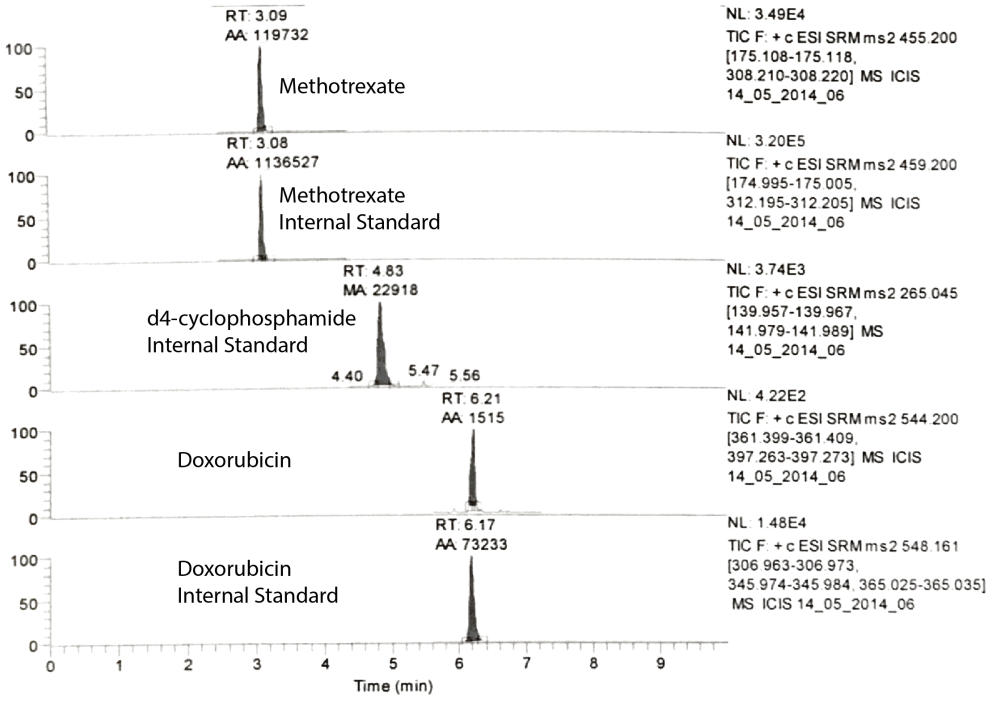
4.4.3. Data analysis and quantification

Chromatogram peaks and analyte calibration curves were integrated using the ICIS algorithm of Xcalibur™ 2.0.7 by Thermo Fisher Scientific. A linear analyte calibration curve was generated using 1/X weighting with six calibration standards prepared at the following concentrations CP – 0.086, 0.43, 0.86, 1.72, 3.44, and 5.16 ng/L; IF – 0.055, 0.28, 0.55, 1.11, 2.21, and 3.32 ng/L with the internal standard (d4-CP) at a constant level of 5 μg/L. For each analysis sequence, calibration standards were bracketed around a maximum of eight unknown samples. Mobile phase blanks with and without d4-CP were used to check for carryover and the purity of the internal standard and the instrument was initially run with pure MeOH prior to each analytical run. Calculations were performed using area ratios of CP and IF on the IS (d4-CP). Recovery of IS d4-CP was ~20% greater for APCI (+) than HESI (+), the lower HESI wastewater recoveries were perhaps due to matrix effects associated with the nature of the samples, signal suppression is significantly more pronounced in HESI than for APCI. It has been proposed that ion suppression is a manifested effect

of competition for ionisation caused by the occurrence of non-volatile solutes in complex extracts, rather than the loss of charge on the analyte due to gas phase reactions that may occur in APCI (King et al., 2000). APCI provided a better overall recovery but with slightly higher instrumental limits of detection (LOD). MDLs for CP ranged between 0.03 - 0.12 ng/L, and between 0.05 - 0.09 ng/L for IF.

A specific method was developed for the determination of other anticancer drugs, methotrexate (MT) and doxorubicin (DOX) with internal standarisation using deuterated methotrexate and doxorubicin. The most successful combination was found to be Strata-X-solid phase extraction cartridge conditioned and loaded with formic acid (0.1%) and EDTA (3%), followed by a 30% methanol clean-up. After the pre-concentration step the cartridge was dried and eluted with 10mL of HPLC grade methanol. The elute was concentrated under N_2 and reconstituted in 500µL of mobile phase. DOX and MT tuning was carried out by directly infusing standards (~1mg/L) into the HESI source and MRM transitions, tube lens voltage and collision energies were recorded. The most abundant ions were identified and automatically optimised for both DOX, MT and their deuterated internal standards. The mass spectrometer was operated in highly selective reaction monitoring (HSRM) mode, significantly reducing matrix noise and improving sensitivity. The selective reaction monitoring chromatograms of DOX, MT, CP and their deuterated internal standards are shown in Figure 4.4.3.

Figure 4.4.3. Selective reaction monitoring chromatograms of DOX, MT, deuterated-DOX, deuterated-MT and deuterated-CP (d4-CP)


RT: 3.09
AA: 119732
Methotrexate
NL: 3.49E4
TIC F: + c ESI SRM ms2 455.200 [175.108-175.118, 308.210-308.220] MS ICIS 14_05_2014_06
RT: 3.08
AA: 1136527
Methotrexate
Internal Standard
NL: 3.20E5
TIC F: + c ESI SRM ms2 459.200 [174.995-175.005, 312.195-312.205] MS ICIS 14_05_2014_06
RT: 4.83
MA: 22918
d4-cyclophosphamide
Internal Standard
4.40
5.47
5.56
NL: 3.74E3
TIC F: + c ESI SRM ms2 265.045 [139.957-139.967, 141.979-141.989] MS 14_05_2014_06
RT: 6.21
AA: 1515
Doxorubicin
NL: 4.22E2
TIC F: + c ESI SRM ms2 544.200 [361.399-361.409, 397.263-397.273] MS ICIS 14_05_2014_06
RT: 6.17
AA: 73233
Doxorubicin
Internal Standard
NL: 1.48E4
TIC F: + c ESI SRM ms2 548.161 [306.963-306.973, 345.974-345.984, 365.025-365.035] MS ICIS 14_05_2014_06
100
50
0
0 1 2 3 4 5 6 7 8 9
Time (min)

5. Discussion of results

5.1. Paper I

After some six decades of worldwide use and increasing developments in cancer therapy, it is timely to review the fate and occurrence of the accumulative number of anticancer drugs available and likely to be present in the aquatic environment. Furthermore, there has been growing concern within the scientific community to better understand the fate of these chemicals in STPs and receiving waters. Collated data on concentrations in hospital wastewaters, STP influents and effluents and receiving waters indicate that some anticancer drugs are poorly degraded during treatment and infiltrate into the environment on both a regional and global scale and hence exhibit a ubiquitous environmental occurrence. Additionally accurate consumption of 65 anticancer drugs in a NW England hospital survey along with their physical-chemical profiles gives insight into their partitioning and fate within aquatic systems. Where the anticancer drugs remain dissolved in the aqueous effluent is fundamental for chemical breakthrough from the STP and their occurrence in surface waters. Of the 65 anticancer drugs in use, approximately twelve drugs are recognised here as being sufficiently persistent to warrant inclusion in environmental screening programmes. Concentrations measured in surface waters for these chemicals are well below the EC_{50} values reported for a wide range of aquatic organisms. Despite the effects of anticancer drugs on aquatic biota being poorly understood a limited number of studies report 5-FU (5-Fluorouracil) as the most concern with ecotoxicity tests categorising the drug as 'very toxic to aquatic organism' (EC_{50} <<1mg/L). However, only limited data is available on ecotoxicity testing for the anticancer agents and many assessments were made on QSAR derived EC_{50} values which doesn't examine their low dose, long term exposure in the environment that is likely to cause subtle

effects due to the high cytotoxic potency of the agents. Furthermore this study has highlighted mitotane (o,p'-DDD) as an additional environmental threat with a log k_{ow} > 3 (with a corresponding high k_{oc} value) indicating a potential for sorption to particle matter, bioconcentration in fish and dispersion to farmland following retention in the sludge at STPs.

5.2. Paper II

Previously, the occurrence of anticancer agents in hospital wastewaters has received considerable attention and various studies have contributed to the current knowledge regarding their concentrations in hospital effluents. Method detection limits have considerably improved over the past few decades and an increasing number of publications have arisen due to analytical advances. Feasibly the few previously undetected anticancer agents could be present in wastewater influents and effluents, however at levels below the methods detection limits. This paper presents new data on the occurrence of CP and IF within the influent and effluent wastewaters of 14 STPs in England, using a previously published analytical method with LODs of 0.12 ng/L and 0.09 ng/L, respectively. CP was frequently detected above the LOD (0.12 ng/L) whereas IF was detected in only 2 of the sampled STP effluents above the LOD (0.09 ng/L). Furthermore, this study shows a significant difference when comparing the influent and effluent concentrations of CP for STPs which operated a tertiary treatment process. For CP, this is important as it is the first study that demonstrates the ability for CP to be reactivated from a metabolite complex (conjugate) to the parent form of the chemical, consequently increasing the output concentration of CP in STPs. Additionally this study evaluates CP in a river catchment study, showing its occurrence in surface waters and accumulation in rivers downstream of the STPs.

This significant finding raises awareness of the environmental concern for CP, especially its implications during periods of low flow.

5.3. Paper III

Removal processes (degradation, partitioning and sorption to sludge) act as major sinks for micropollutants during sewage treatment processes. The performance of STP processes in removal of CP and IF are crucial for understanding the fate, occurrence and risk they pose to the aquatic environment. It is well appreciated that STPs are major contributors of CP and IF to the aquatic environment. The structurally similar anticancer agents possess very similar physical-chemical properties and their removal is expected to be comparable, however their difference in occurrence may be more related to the drug use and their proximity to hospitals than their removal efficiency. Comparison of the treatment efficiency for two tertiary STPs revealed that the elimination of CP was very poor and effluent concentrations for both plants were frequently greater than the influent concentrations. IF was not detected in this study, which is reflective of its low consumption at the contributing hospitals. Reactivation of CP (presumably a glucuronide conjugate of CP) to the parent analyte is shown in all three stages of treatment, but greatest reactivation occurs during secondary treatment whereby the input conjugate is transformed into the original compound.

5.4. Paper IV

CP has been found in to be ubiquitous in sewage influents, effluents and receiving waters. This paper investigates the distribution and levels of CP in the environment. Samples were collected in a defined river basin in NE England with well documented hydrological information. The results demonstrate, that CP and IF are detected in the River Aire and River Calder and surprisingly at similar concentrations (average ~ 1 ng/L). This study is the first for this drug classification to compare real environmental

data to modelled data. Based on modelling and our best fit model inputs we identified a moderate correlation to the measured data. Discrepancies occurred at the lower end of the River Aire and Calder where CP spiked above the predicted modelled concentrations. Indicative that an increased river flow doesn't essentially cause CP to be diluted and instead illustrates that CP is accumulating within the catchment especially since the spike occurs far from a sewage effluent discharge. The cumulative effects of the sum of risk quotient values for the top priority anticancer agents within this catchment showed no risk in the River Aire or Calder, however long term studies for these pollutants are needed to define the environmental stress produced by their continuous exposure and induction.

6. Conclusions

The compounds investigated in this thesis are examples of two widely used anticancer agents both nationally and internationally. However, consumption data for the broad class of anticancer drugs combined with rates of human metabolism provide an assessment of the likelihood of occurrence of these drugs in wastewaters and receiving waters. This approach, taking into account the environmental reactivity and partitioning to particulate matter in wastewater, provides a qualitative insight into their likely survival during wastewater treatment and ability to be discharged to river water. Only a small group of anticancer drugs from the 10s of chemicals in use are likely to be present in final treated effluent, although a significant number partition appreciably to particulate matter and are likely to end up in sewage sludge. Sludge provides a further route for the environmental dispersal of these chemicals, particularly if the sludge is subsequently applied to agricultural land although their reactivity and half-lives need investigating in sludge. This thesis has demonstrated the apparent limitations in predicting environmental concentrations based on consumption data and wastewater flow rates only. Predicted environmental concentrations for some of the anticancer drugs should therefore be treated with caution. For example, environmental water samples of CP contained levels ~2 to 10 fold lower than that predicted for influent and effluent wastewaters. CP has very low rates of degradation and elimination from wastewater streams in STP and this is likely to be mirrored for other similar, highly water soluble anticancer drugs. The fate of CP in STPs is confounded by the apparent positive mass balance whereby concentrations and hence loads increase as wastewater passes through the various treatment processes. Akin to some other pharmaceuticals, a fraction of CP is likely to enter a STP in raw influent as a partially metabolised form, which is subsequently transformed back to the parent

CP molecule, most likely through the action of microbes. This phenomenon may confound modelling predictions of river water concentrations if raw influent loads are used as the emission estimates, as these are likely to be biased low. Further measurement work is required in order to determine this metabolite and the process by which it is transformed back to CP (both biotic and abiotic processes). The physical-chemical profiles of CP and IF indicate that these chemicals remain in the dissolved phase and will not be lost from wastewater streams either through volatilisation or through particle settling and are therefore discharged in the STP effluents to recipient waters. The recipient rivers have CP and IF concentrations present at approximately 1 ng/L, and are still detectable and quantifiable despite dilution far from STP effluent discharge points. CP shows accumulation downstream within river basin studies and this highlights the persistence of this chemical in rivers , with ongoing use/discharge of this drug likely to maintain levels in river and estuarine waters for the foreseeable future. While this thesis has chiefly focused on CP and IF - certainly with regards to environmental measurements - the fate of another 10-15 drugs that are in common use and possess similar physical-chemcial properties to CP and IF, is now warranted and should be subject to both human and ecological risk assessments. In the case of human risk assessment, exposure occurs through the abstraction of river water as a potable water supply. The elimination of these drugs during water treatment processes is likely to be negligible given the lack of elimination during secondary and tertiary wastewater treatment, although remediation techniques such as activated-carbon filters need testing for this group of drugs. The river water concentrations measured in this thesis generally fall below those considered to cause harm in standard toxicity assays (i.e. LC_{50} or EC_{50} values). Given the genotoxic nature of many of these chemicals then alternative toxicity endpoints

may be warranted, as a defined safe-level is unknown. For example, genotoxicity assays performed on sentinel aquatic species (or their relevant tissues) may be called for. The ongoing use and consumption of chemotherapy agents in the UK and other developed nations will continue and may actually increase (given the ageing populations in these countries) in the next few decades. It is therefore prudent to initiate some type of monitoring campaign either for STP effluents or in receiving waters (particularly in catchments where river water is abstracted as a potable water supply). This type of programme, coupled to ecotoxicity assays, would prove useful for setting environmental quality standards for some of these chemicals.

7. Future research needs

Research on the environmental fate and impact of potent pharmaceuticals like anticancer drugs is currently in its infancy. With regards to basic water pollutants like nitrate, phosphate, suspended particulate matter or heavy metals, there is very little published literature regarding the environmental occurrence and fate of these chemicals. This thesis has largely focused on just two of these compounds, CP and IF, but has highlighted their widespread occurrence and persistency. Areas for additional research include:

- There is a need for a robust multi-compound screening method able to detect 'priority' anticancer agents in wastewater influents, effluents and receiving waters at concentrations ranging from sub to low ng/L.
- Regional surveys on consumption need to be carried out to prioritise those compounds with the highest consumption and longest environmental half lives for environmental screening surveys.

In addition to this, there are some anticancer agents that have relatively low consumption but possess physical-chemical properties that demonstrate likely removal from STPs via sorption to sludge. Some of these chemicals, such as mitotane, have significant environmental concern. Mitotane (or *o,p'*-DDD) is a chemical related to the DDT 'family' (dichlorodiphenyltrichloroethane) and hence can be considered as a persistent organic pollutant, possessing a log K_{ow} >5 and a very long environmental half-life (e.g. >6 months in soil/sediment) Mitotane is applied during end-stage chemotherapy in specialist oncology units in relatively large doses and in consequence the STPs serving these units may contain a significant load of this chemical in the sludge, where further problems are introduced if this sludge is

subsequently applied to agricultural land. There is therefore a research need to identify those anticancer drugs which are most likely to partition appreciably to sludge and persist in this matrix, with studies aimed at addressing the fate of these chemicals following sludge application to pasture/agricultural land.

From the STPs investigated in this thesis - several with tertiary treatment processes – elimination of CP and IF was found to be very low (< 20%). The chemicals stay dissolved in the aqueous phase, resist biodegradation, UV degradation and other bio-transformation processes. A further stage of tertiary treatment would be a beneficial addition to STPs in the UK, not only to remove this sub-group of chemicals but also to target other pharmaceuticals that have similar physical-chemical properties. Initial research has shown that advanced oxidation processes e.g. UV-light coupled to an oxidative agent like H_2O_2, may be sufficient to degrade CP and other similar anticancer drugs from wastewater effluents (Kim et al., 2009).

The whole concept of anticancer drug fate and behaviour in the aquatic environment is an important area of future research and screening campaigns for other chemicals should be carried out in defined river basins. Along with aqueous degradation and photodegradation, studies could be focussed on realistic freshwater scenarios using environmentally relevant concentrations of the compounds. This could lead to valuable information on anticancer drugs degradation and the accumulation of anticancer drugs in river systems, particularly important for the river basins that extract potable water downstream of STPs.

There is a lack of data regarding ecotoxicity for the anticancer agents and their effects on sentinel aquatic organisms. Where data does exist, it is expressed as a half maximal effective concentration (EC_{50}) and the reported values are far greater than

the levels found in the wastewater effluents and river waters. The EC_{50} values give a good indication of the drugs potency, but since anticancer agents are cytotoxic in action and cause subtle genetic alterations to cells, their mutagenicity may appear over generations rather than days to weeks, depending on the organism in question. Some studies have derived ecotoxicity endpoints for these agents; however this has been demonstrated at concentrations far beyond the levels observed in river water. These agents have low dose toxicity and may still act negatively in the environment at these sub-low ng/L concentrations and insight into their long term effects would further focus environmental screening programmes.

A final thought for further research would be to develop a passive sampler to monitor levels in freshwater systems and investigate the temporal trends (e.g. periods of low flow) of the contaminants. A passive sampler would also provide additional information on the diffusion and spatial distribution across catchment waters and highlight the effect of individual STPs on river water concentrations.

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Paper I

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Prioritising anticancer drugs for environmental monitoring and risk assessment purposes

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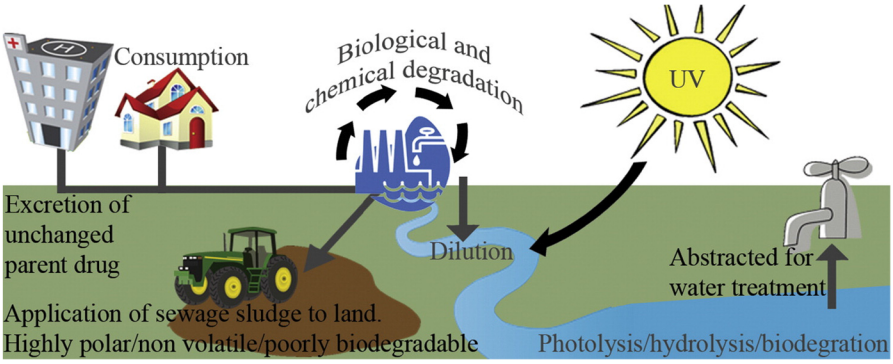
HIGHLIGHTS

- We surveyed regional hospitals to get accurate consumption data for anticancer drugs.
- Drugs were systematically ranked based on consumption, behaviour and fate.
- A shortlist of 18 drugs is likely to be of environmental concern.
- 12 anticancer drugs can 'breakthrough' to receiving waters.
- 6 anticancer drugs partition appreciably to sewage sludge and may persist.

GRAPHICAL ABSTRACT



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ABSTRACT

Anticancer drugs routinely used in chemotherapy enter wastewater through the excretion of the non-metabolised drug following administration to patients. This study considers the consumption and subsequent behaviour and occurrence of these chemicals in aquatic systems, with the aim of prioritising a selection of these drugs which are likely to persist in the environment and hence be considered for environmental screening programmes. Accurate consumption data were compiled from a hospital survey in NW England and combined with urinary excretion rates derived from clinical studies. Physical–chemical property data were compiled along with likely chemical fate and persistence during and after wastewater treatment. A shortlist of 15 chemicals (from 65) was prioritised based on their consumption, persistency and likelihood of occurrence in surface waters and supported by observational studies where possible. The ecological impact of these 'prioritised' chemicals is uncertain as the measured concentrations in surface waters generally fall below standard toxicity thresholds. Nonetheless, this prioritised sub-list should prove useful for developing environmental screening programmes.

1. Introduction

There is growing concern about the presence of pharmaceuticals in the wider aquatic environment. Common 'over the counter' and prescription medicines as well as veterinary medicines are increasingly reported in waste and surface waters in the scientific literature (Jones

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et al., 2005; Buerge et al., 2006; Moldovan 2006; Yin et al., 2010; Martín et al., 2011; Gómez-Canela et al., 2012). However, anticancer drugs used in chemotherapy have received less attention but have high pharmacological potency and possess fetotoxic, genotoxic and teratogenic properties and can induce subtle genetic and cell cycle changes in aquatic fauna and flora under chronic exposure (Johnson et al., 2008; Rowney et al., 2009).

Due to the improvement in detection limits (from <10 ng/L in 1998 to >1 ng/L in 2011) to quantify anticancer drugs with liquid chromatography tandem mass spectrometry (LC–MS/MS) some of these chemicals have been reported in hospital waste effluents, influents/effluents in sewage treatment plants (STPs) and river water, in a small but growing number of studies (Aherne et al., 1990; Castiglioni et al., 2005; Buerge et al., 2006; Mahnik et al., 2006; Garcia-Ac et al., 2009; Kovalova et al., 2009; Yin et al., 2010; Llewellyn et al., 2011; Martín et al., 2011). The concern over these substances is their occurrence in freshwater systems which are then abstracted as a potable water supply, hence presenting a risk of human exposure, as well as posing a wider risk to freshwater and estuarine habitats (Rowney et al., 2009).

Anticancer drugs are classified under antineoplastic and immunomodulating agents (class L) using the Anatomical Therapeutic Classification (ATC) system. Based on the chemical structure and therapeutic properties they are further subcategorised into five groups; L01A: alkylating agents; L01B: antimetabolites; L01C: plant alkaloids & other natural products; L01D: cytotoxic antibiotics & related substances, and L01X: other antineoplastic agents which relate to their mode of action. Chemotherapy is correctly described as cytotoxic therapy, and refers to the use of drugs to kill or inhibit the growth of cancer cells. Most chemotherapy drugs act as cytotoxic agents by causing damage to deoxyribonucleic acid (DNA) or prevent chromosomal replication by disrupting critical cell processes, which leads to cell death (apoptosis) (Caley and Jones 2012). There are other treatments (cytostatic agents) that do not kill cancer cells and work by stopping cancer cell replication/division and arresting cells in a specific phase of their cell cycle. Trastuzumab (common name: Herceptin) is an example of a cytostatic agent that has high consumption, in France (Besse et al., 2012). Once cancerous cells are arrested and synchronised they can be targeted with a cytotoxic agent (Caley and Jones 2012).

Currently, there are over fifty anticancer drugs being used routinely in chemotherapy in the UK. In general, many of these compounds are polar, water soluble and non-volatile with principle sources including wastewater through point release as hospital effluents as well as diffusive release from domestic dwellings from cancer patients (non-hospital bound or 'outpatients') undergoing chemotherapy medication. Therefore, STP discharges are considered as the main source of anticancer drugs to the aquatic environment (Kummerer 2001; Rowney et al., 2009). However, in countries where on-site wastewater treatment systems (i.e. septic systems) are extensively used the diffusive release from domestic dwellings may provide significant entry of anticancer drugs into the environment (Stanford and Weinberg 2010; Du et al., 2014).

Some of these drugs are not fully metabolised and are poorly biodegradable and therefore can resist biological as well as physical removal processes during wastewater treatment (Johnson et al., 2008). Some of these chemicals could be considered to be semi-persistent with ongoing release into the environment (Daughton 2002; Jones et al., 2005). Given that many of the drugs possess a similar pharmacology then it is plausible that they may act additively once in the environment, possibly enhancing their overall cytotoxicity and increasing the risk to aquatic organisms (Lambert and Lipscomb 2007).

The aim of this study was to generate a shortlist of anticancer drugs (from the many drugs in use) that are likely to have relevance with regard to their actual occurrence and impact on the wider environment. By following a systematic methodology examining consumption, excretion and chemical fate we are able to generate a shortlist of priority chemicals that can then be used to inform future screening programmes and/or targeted risk assessments.

2. Methods

To generate a shortlist of priority anticancer drugs, a systematic, stepwise approach was taken which is outlined in Fig. 1. Contemporary use and consumption data of anticancer drugs were obtained for 31 hospitals operating a range of specialist and/or non-specialist oncology units in NW England. Drugs were ranked according to their annual use and then grouped according to their rates of metabolism. Low metabolism assumes that a high percentage of the consumed parent drug is lost via excretion (via urine and faeces) to the wastewater system. Chemical fate in wastewater was then undertaken using chemical property estimation and fate models including the use of SPARC (http://archemcalc.com/sparc/) and the EPI-Suite models (http://www.epa.gov/oppt/exposure/pubs/episuite.htm) and supported by empirical studies to ascertain partitioning (i.e. between the dissolved aqueous phase and suspended particulate matter) and the susceptibility of a drug to undergo transformation/degradation. For this step efforts were made to assess and select the most appropriate physical–chemical property data, particularly aqueous solubility and K_{ow} or D_{ow} values. Furthermore, estimates of key abiotic or biotic loss processes were undertaken. Drugs could then be grouped according to those most likely to exist in the dissolved phase but with sufficient persistency to reach surface waters (via treated effluent) and those partitioned strongly to particle matter and sufficiently persistent to be retained in sewage sludge. Those drugs considered to be present in the final effluent were then assessed with regard to their likely release to receiving water

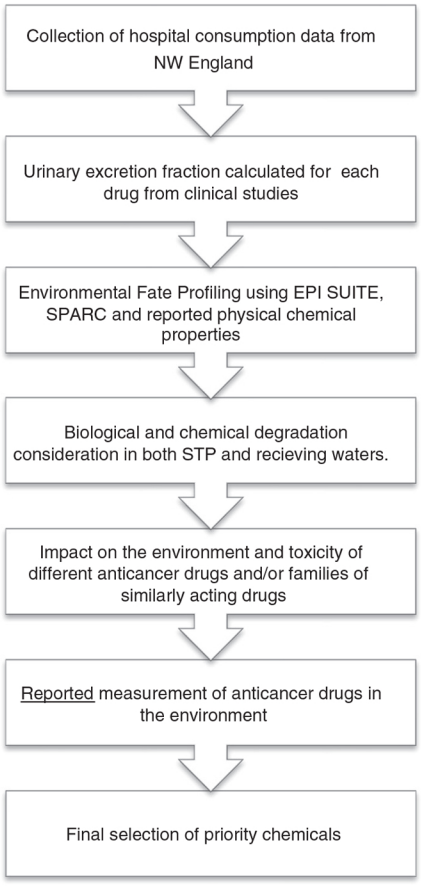

Fig. 1. Schematic representation of the methodology used to select priority chemicals.

according to their predicted environmental concentrations (PEC) and supported by measurements reported in the literature. Environmental toxicology data for specific anticancer drugs were also reviewed to ascertain their likely impact on key aquatic organisms that represent different trophic levels. The inclusion or exclusion criteria are best illustrated in Table S5 (Supplementary data) (see later sections in the Results and discussion) which reports in full the PECs of the anticancer drugs in STP effluents and surface waters through use of Eq. (1), with PEC (μg/L) given by:

$$\mathrm{PEC} = \frac{Cons \times Exc \times Biodeg}{HDil \times 10} \quad (1)$$

where *Cons* is the per capita consumption (μg/capita/d) of an anticancer drug in a defined zone; *Exc* is the percentage of the original drug that is excreted; *Biodeg* is the percentage of intact drug after STP biodegradation; and *HDil* is the head dilution in litres expected in the STP (usually 200 L/capita/d) with a factor of 10 accounting for a 10-fold dilution expected for river waters. Based on this assessment, the anticancer drugs were systematically ranked and other factors such as lack of knowledge, or specific chemistry for a related group of drugs were considered when prioritising a shortlist of drugs.

While this approach is based on UK hospital consumption, a wide range of hospitals (of varying sizes) and oncology units were included. As such the data are therefore likely to capture consumption typical of countries in Western Europe, North America and other developed regions of the world. The following sections detail the findings based on the various steps outlined in Fig. 1.

3. Results and discussion

3.1. Hospital consumption

Consumption data was obtained for hospitals within the NW of England and used to generate an accurate annual usage dataset for the 65 anticancer drugs listed by these hospitals. The National Health Service (NHS) trusts within the region are listed in Table S1 (Supplementary data) with their corresponding annual consumption (g/yr) and calculated per capita consumption (μg/cap/d). The survey lists the collective consumption of each chemical administered at all hospital and outpatient sites contained within the NHS trust with the exception of the Greater Manchester & Cheshire cancer network and the Clatterbridge Centre of Oncology. The Greater Manchester and Cheshire cancer network (Table S1) contains 11 trusts; five trusts (Trafford Healthcare NHS trust, Royal Bolton Hospital NHS foundation trust, Salford Royal NHS foundation trust, Mid Cheshire Hospitals NHS foundation trust and Wrightington, Wigan and Leigh NHS foundation trust) have been combined to give the consumption for this region. The highest consumed anticancer drugs from Table S1 are capecitabine > cyclophosphamide > hydroxyurea > 5-fluorouracil > imatinib > gemcitabine. These data complement a French study conducted in the city of Lyon (Besse et al., 2012). An unpublished study at the University Hospital in Aachen (Germany) also highlighted dacarbazine, methotrexate and cytarabine as highly consumed chemicals (i.e. > 178 g/yr) (Kovalova 2009).

Chemotherapy drugs are normally administered in cycles of one or more days followed by a period for 'normal' tissue to recover (as non-cancerous cells have a greater capacity for repair than tumour cells, the repeated cycles decrease the tumour population with time (Caley and Jones 2012)). The implications for this type of dosing to cancer patients are that 'emissions' of the unmetabolised or partially metabolised drug are continuous and not restricted both spatially or temporally, ensuring continuous input to wastewater. Anticancer drugs are sometimes applied in combination, for example the cisplatin–etoposide (PE) regime is an example of combinational chemotherapy used to treat different types of lung-cancer where typically cisplatin (75 mg/m^2) and etoposide (300 mg/m^2) are given in a 4:1 ratio over three consecutive days (Ardizzoni et al., 1999). This relationship can be seen in Table S1, where consumption data from the University Hospital of Morecambe Bay show that the consumption of cisplatin is proportional to etoposide in this given ratio.

3.2. Drug metabolism and excretion

The combined excretion of pharmaceutical ingredients via urine and faeces is considered the primary route by which the active pharmaceutical enters the environment. Table S2 shows the average % urinary excretion of the parent pharmaceuticals for the 65 anticancer drugs surveyed in NW hospitals and calculated from clinical studies. There is considerable variability in the pharmacokinetics of an administered drug and the urinary excretion rates show strong inter-patient variation (Kovalova 2009). Nonetheless, the average percentage urinary excretion of the unchanged parent drug ranged from negligible to >75%. From Table S2, methotrexate and pemetrexed show the lowest metabolism with the highest average urinary excretion rates. Using the available consumption data and corresponding urinary excretion rates then these provided an initial evaluation for the likelihood of cytotoxic drugs to be present in the wider environment (Besse et al., 2012). The following drugs were not considered further due to low consumption and/or excretion: chlorambucil, busulfan, lomustine, tioguanine, cladribine, vinblastine, vincristine, trabectedin, idarubicin, mitoxantrone, gefitinib, dasatinib, temsirolimus and everolimus. Geographic difference is expected to be an important factor, however, a different European study showed negligible PECs for these compounds due to low consumption and/or excretion (Besse et al., 2012). The monoclonal antibodies L01XC (rituximab, trastuzumab, alemtuzumab, cetuximab, bevacizumab) were also not included in the next stage of assessment due to the lack of data on their physical–chemical properties and urinary excretion rates, although they may require future evaluation due to their high consumption (see Table S1) (Besse et al., 2012).

In general there is a lack of data concerning the faecal excretion of anticancer drugs, although most display negligible faecal excretion of the unchanged parent drug. Of the 65 anticancer drugs studied imatinib, capecitabine, sorafenib, mitotane, paclitaxel, 5-fluorouracil, lapatinib, sunitinib and erlotinib may be excreted via faeces and will be dealt with in a later section (Section 3.7).

3.3. Building a fate profile based on physical chemical properties

Table 1 presents the physical–chemical properties of a wide range of anticancer drugs and their predicted loss from wastewater during the sewage treatment process. Building a physicochemical profile for each drug allows their partitioning and fate within aquatic systems to be predicted to some extent. The fate of the chemical entering an STP depends on both the nature of the chemical and the treatment process; anticancer drugs may sorb to sludge, undergo volatilisation or remain in the dissolved phase in the aqueous effluent. The latter is most important for chemical breakthrough from the processing plant and occurrence in surface waters. Sorption to suspended particulate matter and deposition with sludge would be an important route of removal for those compounds with high K_{ow} and/or K_{oc}.

Many of the chemicals listed in Table 1 are highly polar with high aqueous solubility (i.e. ~10^3–10^4 mg/L), although a wide range is evident (1.07×10^{-4} to 8.66×10^4 mg/L). The presence of ionisable functional moieties indicates that a large number of these compounds are likely to be ionised at environmentally relevant pHs. pKa values are given in Table 1 (largely gleaned from pharmaceutical data or reports) for behaviour in blood (pH 7.4). The presence of two or more carboxylic acid groups on methotrexate, for example, results in successive pKa values of 3.8, 4.8 and 5.6. The corresponding log K_{ow} and log D_{ow} are also presented in Table 1. K_{ow} values, in general, are low (i.e. < log 2)

Table 1
Physiochemical properties of selected cytotoxic drugs and their classification according to the Anatomical Therapeutic Classification (ATC) system. Table S3 in the Supplementary data provides property information for the full list of drugs included in this study.

ATC	Drug name	pKa	Charge at pH 7.4	Weak acid/weak base	Log K_{ow}	Log D_{ow} at pH 7.4	K_{oc}[a]	BCF[a]	Solubility in water (mg/L) at 25 °C[b]
L01AA01	Cyclophosphamide	2.84, 6.00 [a, b]	Neutral	Acid [b]	0.63		44	3	4.00E+04
L01BC06	Capecitabine	8.8 [c]	Neutral	Acid	0.96[b]		8	3	8.23E+02
L01BC02	5-Fluorouracil	7.6–8.0, 13.0 [c, d, f]	Neutral	Acid [b]	−0.93		4	3	1.11E+04
L01XX05	Hydroxyurea	10.6[b]	Neutral	Acid	−1.27		3	3	7.91E+04
L01XE01	Imatinib	8.07, 3.73, 2.56, 1.52[b]	Positive	Base		0.19	16	3	6.48E+01
L01BA01	Methotrexate	3.80, 4.8, 5.6 [c]	Negative	Acid		−1.41	20	3	4.98E+03
L01XA02	Carboplatin	0.24, 3.55[b]		Base	−1.78	0.01	891 [e]		
L01BC05	Gemcitabine	3.6 [d]	Neutral	Base	−1.24		1	3	1.53E+04[c]
L01CB01	Etoposide	9.8 [f]	Neutral	Acid	0.60		19	3	5.87E+01
L01AA05	Ifosfamide	1.45–4.0 [a, b]	Neutral	Base [b]	0.86		51	3	3.78E+03
L01AX04	Dacarbazine	4.42[b]	Neutral	Base	−0.24		15	10	4.22E+03
L01AB02	Treosulfan	12.36[b]	Neutral	Acid	−2.09[b]		1	3	7.00E+04[d]
L01XX23	Mitotane	N/A	Neutral	N/A	6.11[b]		154,882	4989	1.00E−01
L01XE07	Lapatinib	3.80, 7.20[b,c]	Positive	Base		4.72	426,580	1127	9.06E−02
L01CD01	Paclitaxel	11.99	Neutral	Zwitterion [b]	5.25		58,884	750	1.07E−04

[a] Sottani et al. (2008); [b] Mahoney et al. (2003); [c] *The hazardous substances databank*, TOXNET; [d] Kovalova (2009); [e] Lenz et al. (2007); [f] Weylandt et al. (2007).
N/A—not applicable.
[a] Fish BCF (Bioconcentration Factor) predicted with EPI SUITE: linear relationship with K_{ow} does not hold for many compounds with high polarity (see text).
[b] From predicted data.
[c] MSDS (Material Safety Data Sheet) gemcitabine (http://ehs.lilly.com/msds/Gemzar.pdf).
[d] MSDS treosulfan (http://www.medac.de/medac_international/data/SDS/treosulfan_E.pdf).

indicating the propensity of these drugs to remain in the dissolved phase, although there are exceptions to this. For example, lapatinib, mitotane, nilotinib, paclitaxel, vinorelbine, pazopanib, sorafenib and irinotecan, possess log K_{ow} or D_{ow} values that exceed 3, with correspondingly high K_{oc} values indicating a potential for bioconcentration in fish. Since EPI-Suite models were used to derive K_{ow} values then these can only be considered as estimates at best and are unlikely to be accurate for polar molecules containing multiple functional groups. For example doxorubicin (DOX), an anthracycline antibiotic, sorbs to sludge despite its relatively low K_{oc} predicted by EPI-Suite (Mahnik, Lenz et al. 2007). Tetracycline (TET) is structurally similar to DOX and shows similarly high rates of sorption to organic rich particulate material (Leung et al., 2012). Positively charged compounds with log $D_{ow} > 1$ have a strong sorbing tendency to sewage solid surfaces partly by electrostatic interaction. The microorganisms have a negatively charged surface, where a stronger association will occur with a positively charged species than a neutral. Simplified empirical models may underestimate the sorption behaviour in these chemicals (Stevens-Garmon et al., 2011).

Cyclophosphamide (CP) is one of the most widely used cytotoxic drugs and is frequently detected in surface waters. It is therefore appropriate to relate other drugs to CP to give a relative indication of their likely environmental behaviour. For example, CP has a water solubility of 40,000 mg/L and has been detected in hospital effluent at a maximum concentration of 4.5 mg/L (Steger-Hartmann, Kümmerer et al. 1997). Where data are available, the high aqueous solubilities, low vapour pressures and hence very low Henry's Law constants indicate that it is unlikely that these compounds will volatilise at ambient temperatures from surface waters. The vapour pressures for the drugs range from $<10^{-7}$ to 10^{-2} Pa and the Henry's Law constants range from $<10^{-10}$ to 10^{-5} Pa m^3/mol. For CP and similar compounds possessing water solubilities in the g/L range, combined with low log K_{ow} values indicate that the chemicals will remain in the dissolved phase and will not be lost from wastewater streams either through volatilisation or through particle settling.

3.3.1. Dissociation and partitioning in aquatic systems

At environmentally relevant pHs (e.g. 5–9), fifteen of the listed anticancer drugs (Table 1) will be partially or fully ionised. Fig. 2 illustrates the relative proportions of acid–base species as a function of pH for some of the anticancer drugs exhibiting more than one acid or base moiety. More complex sorption related modelling in representative aqueous systems appears necessary to determine whether the drug is removed from the dissolved phase, with the ambient pH influencing the degree of dissociation for ionisable drugs and hence the D_{ow} and D_{oc} values. For example, the particle–water distribution ratio K_d for chemicals containing an amine functional group (hence basic properties) can be affected by changes in pH, although changes over the environmental range of pH appear to have little effect on K_d and hence sorption potential (Hörsing et al., 2011).

Fig. S1 illustrates the range of log K_{ow} values for many of the anticancer drugs. Where relevant, D_{ow} values were then calculated from K_{ow} according to:

$$D_{ow} = K_{ow} + 1/\left(1 + 10^{(pH-pKa)}\right) \quad (2)$$

for acidic compounds, and

$$D_{ow} = K_{ow} + 1/\left(1 + 10^{(pKa-pH)}\right) \quad (3)$$

for basic compounds, with values reported in Table 1. Typically, chemicals with a log K_{ow} over 2.5 and a log D_{ow} greater than 3 can be considered to partition significantly to organic rich particulate matter and hence be removed from the water column particularly during primary sewage treatment. Values of K_{ow} and corresponding K_{oc} should be interpreted with caution as Yamamoto et al. (2009) demonstrated the lack of correlation when plotting the Karickhoff empirical formula ($\log K_{oc} = \log K_{ow} - 0.21$) for pharmaceuticals with experimentally derived K_{oc} values. Furthermore, pharmaceuticals with ionisable functional groups (e.g. carboxylic acids or amino groups) will undergo a range of sorption mechanisms with the existence of electron donating and/or accepting functional groups affecting electrochemical affinity to particle or sediment surfaces (Yamamoto et al., 2009).

Using the K_{ow}/K_{oc} values reported in Table 1 (and illustrated in Fig. S1) (D_{ow} if dissociated at pH 7) then those anticancer drugs likely to show strong tendency for sorption are: lapatinib > mitotane > nilotinib > paclitaxel > vinorelbine > cisplatin > pazopanib, and possess K_{oc} values >5000. Compounds with K_{oc} values greater than 2000 are sorafenib > irinotecan > erlotinib with potential to sorb to sewage sludge. Bendamustine and carboplatin also have moderately high K_{oc}

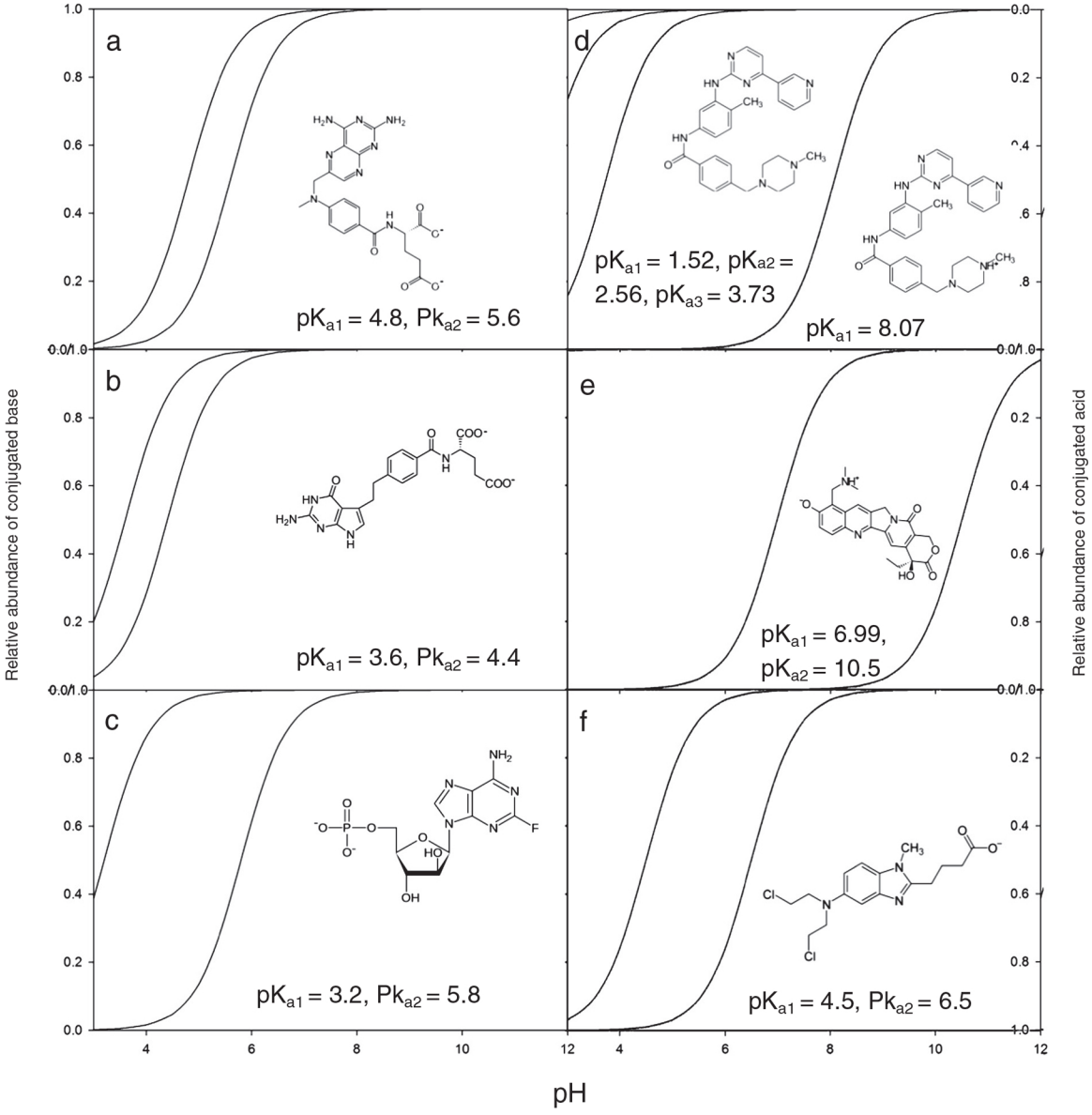

Fig. 2. Calculated proportions of neutral and ionised forms of selected anticancer drugs as a function of pH a) methotrexate, b) pemetrexed, c) fludarabine, d) imatinib, e) topotecan, and f) bendamustine.

values (approx 900) (McCall et al., 1980). Sorption to particulate matter is therefore likely for these chemicals, with a significant fraction likely to be retained in sewage sludge following primary treatment. Depending on their persistence and resistance to biodegradation then these chemicals may eventually find their way to soil if the sludge is subsequently applied to agricultural land. The remaining anticancer drugs have K_{oc} values <500 and are expected to display limited partitioning to suspended particulate matter in STP influent. Some anticancer drugs may enter STPs as conjugated or metabolised entities, for example, cyclophosphamide is a substrate for glutathione S-transferase (GST) which catalyses the conjugation of glutathione (as part of phase II metabolism) (Deenen et al., 2011). Other anticancer drugs such as the platinum-based compounds (e.g. cisplatin), melphalan, busulfan, chlorambucil, cyclophosphamide and anthracyclines are known substrates for conjugation (Deenen et al., 2011). Conjugation raises the potential for the parent chemical to be 'liberated' during the various stages of effluent treatment, possibly through biotic transformation processes, although at present there is little if any evidence of this in the literature.

3.4. Degradation and environmental half-lives

3.4.1. Biodegradation

Following release with wastewater streams biological degradation by micro-organisms (aerobic or anaerobic), particularly in the secondary stage of sewage treatment, may be a significant loss process for some anticancer drugs. Degradation will be dictated by both physical–chemical properties of the drug and the characteristics of the treatment plant. Biodegradability tests such as the closed bottle test (CBT) or Zahn–Wellens test (ZWT) are usually carried out with the initial quantity of the test chemical in the order of mM, with the drug serving as the carbon source. Results from these tests should therefore be viewed with caution as levels of anticancer drugs in wastewater are likely to be much lower than those used in these tests. Cyclophosphamide (StegerHartmann et al., 1996; Steger-Hartmann et al., 1997; Kiffmeyer et al., 1998; Kümmerer et al., 2000; Buerge et al., 2006), ifosfamide (StegerHartmann et al., 1996; Kümmerer et al., 1997, 2000; Buerge et al., 2006), vinblastine (Al-Ahmad and Kummerer

2001), vincristine (Al-Ahmad and Kummerer 2001) and mitoxantrone (AlAhmad et al., 1997) have been shown to exhibit poor biodegradability, and a summary of these tests is presented in Table S4. For example, the incubation of CP in activated sludge resulted in no observed degradation after 24 h. After prolonged incubation for 39 days, only 17% of CP was removed, indicating the stability of this drug (Steger-Hartmann, Kümmerer et al. 1997) and CP showed no degradation for environmentally relevant concentrations (ng/L) over a 24 hour period (Buerge et al., 2006). Another study investigated the removal of trace levels of CP from sewage waters by using nanofiltration and reverse osmosis, however, CP was not sufficiently removed in these tests although a system combining both techniques may prove successful in eliminating CP (Wang et al., 2009).

Other compounds such as treosulfan (AlAhmad et al., 1997), methotrexate (Kiffmeyer et al., 1998), pemetrexed (EMA 2006), gemcitabine (Kummerer and Al-Ahmad 1997; Kümmerer et al., 2000), capecitabine (Straub 2010), cytarabine (Kummerer and Al-Ahmad 1997; Kiffmeyer et al., 1998), doxorubicin (Mahnik et al., 2007) and epirubicin (Mahnik, Lenz et al. 2007) have been shown to be biodegradable to some extent (see Table S4), although in many cases biodegradation results are conflicting (Henschela et al., 1997). For example, several studies indicate that biodegradation of 5-fluorouracil is negligible (Kummerer and Al-Ahmad 1997; Straub 2010) yet others show significant biodegradation, with 94% biodegradation demonstrated over a 14 day period in a laboratory-scale activated sludge plant (Kiffmeyer et al., 1998; Mahnik et al., 2007; Straub 2010). In this case, ~15% of 5-FU was removed in the first 24 h, followed by a marked increase to almost complete degradation between 24 and 48 h. However, microorganisms in STPs will not have an adaptation period of several days in order to achieve the high degradation efficiencies observed in the above tests and therefore environmental degradation rates are likely to be low and reflect those measured in the initial stages of these experiments (Kiffmeyer et al., 1998). Since there is a paucity of empirical biodegradation studies, other anticancer drugs listed in Table S4 were assessed based on modelled biodegradation using the EPI-Suite model, STPWIN (v. 4.1.) with consideration to other anticancer drugs in the same ATC classification or possessing a similar structure. In general, most anticancer drugs have low biodegradability and it can be assumed that biological degradation in surface waters will also be negligible (Kosjek and Heath 2011). Furthermore, biotransformation products or metabolites may also need consideration with regard to their occurrence in surface waters and possible risk. For example, methotrexate has been shown to degrade into the active metabolite, 7-hydroxymethotrexate, which acts via the same cytotoxic mechanism as methotrexate but with a lower potency (Kiffmeyer et al., 1998). 7-Hydroxymethotrexate does not appear to undergo further biodegradation and therefore may persist for longer periods than the parent chemical.

3.4.2. Abiotic degradation: photo-transformation and hydrolysis

Anticancer drugs that exhibit significant light absorption within the solar light spectrum may be subject to direct photolysis in surface waters. Examples include cisplatin and methotrexate that exhibit UV maxima absorbance values of 318 nm and 298 nm (within the solar UV-B range) respectively, and are likely to undergo direct photolysis (Kiffmeyer et al., 1998).

Cyclophosphamide and ifosfamide show negligible light absorption in the solar wavelength range (Buerge et al., 2006). Direct photolysis studies to date generally use a lamp with appropriate filters to simulate sunlight with light irradiance approximately equivalent to midsummer sunlight in California, and the studies do not account for light attenuation in the water column and other site-specific factors (Lin and Reinhard 2005). UV irradiation experiments showed that only 10% of an aqueous solution of cytarabine was degraded after 2 h, with a quantum yield of <0.01 (Ocampo-Pérez et al., 2010). UV lights fitted as tertiary treatment in some STPs may result in some loss of cytotoxic drugs. Indirect photolysis involving the transformation of the drug through a reaction with photochemically derived species like the aqueous hydroxyl radical (OH•) is perhaps more relevant for some anticancer drugs present in surface waters. The photolysis rates in river water are generally faster than those in MilliQ laboratory water, with the accelerated rates attributed to photosensitisation by dissolved organic carbon (DOM) (Lin and Reinhard 2005). Cyclophosphamide and ifosfamide were degraded at faster rates in irradiated lake water, with increasing OH• concentrations enhancing photodegradation rates (Buerge et al., 2006). However, depending on the structural properties, DOM can retard the reaction by competing for photo-radicals and acting as an optical filter or quencher. The latter may explain slower rates of photodegradation rates in river water compared to MilliQ (Lin and Reinhard 2005). UV/H_2O_2 systems were adequate to degrade cytarabine although toxicity assays showed that the photodegradation products were more toxic than the parent chemical (Ocampo-Pérez et al., 2010).

Hydrolysis may also provide a transformation route for cytotoxic drugs. Hydrolysis rates are expressed in terms of the acid–neutral- and base-catalysed hydrolysis rate constants and drugs containing ester and amide functional groups are particularly susceptible to degradation via hydrolysis. To investigate this process, the rate constants for the base-driven hydrolysis for 11 relevant anticancer drugs (cyclophosphamide, ifosfamide, vinblastine, vincristine, vinorelbine, etoposide, paclitaxel, docetaxel, cisplatin, topotecan and irinotecan) were generated by SPARC (version 4.5) and reported in Table S4. The SPARC derived hydrolysis rate shows that at pH 7, the percent loss of parent compound is negligible in all cases, however, at pH 8.1 (the pH of seawater, relevant for coastal waters), vinblastine, vincristine, vinorelbine, paclitaxel and irinotecan undergo significant hydrolysis with losses ranging over ~54–77% (for a 5 day period). This range is an estimate at best but does highlight the relative susceptibility of these drugs to be lost by this process, particularly at higher pHs.

Since the hydrolysis of most anticancer drugs at pH 7 appears to be negligible, biodegradation and photolysis are likely to be the primary degradation routes for the drugs listed in Table S4. In addition to hydrolysis and photolysis, the calculated rate constants for the reaction between dissolved ozone (used in water treatment processes) and cyclophosphamide suggest that ozone reactions will play a minor role in the degradation of this chemical. For other chemicals containing amino groups, such as methotrexate, then reaction with ozone can be rapid (Garcia-Ac et al., 2010). Furthermore, sorption to sediments generally reduces the rates of hydrolysis for acid- or base-catalysed reactions but neutral reactions appear to be unaffected by sorption, so particle-sorbed compounds may undergo some transformation by this process.

3.5. Ecotoxicity and genotoxicity (impact on the environment)

The effects of anticancer drugs on aquatic biota are not well understood, but attempts have been made to assess human risk based on exposure through consumption of drinking water containing anticancer drugs in the low ng/L range, i.e. the concentrations observed in surface waters (see next section) (Johnson et al., 2008; Rowney et al., 2009). The EU-Directives 93/67/ECC (EC 1996) classifies chemicals according to their EC_{50} values (whereby $EC_{50} < 1$ mg/L is deemed 'very toxic to aquatic organisms'; 1–10 mg/L are 'toxic to aquatic organisms' and 10–100 mg/L are classified as 'harmful to aquatic organisms' and chemicals with an EC_{50} above 100 mg/L are not classified). Predicting the effects of chemicals on biota can be attempted using Quantitative Structure Activity Relationships (QSAR), or measured using a range of acute and chronic ecotoxicity tests, that have been conducted for algae, *Daphnia* and fish, i.e. different trophic levels of freshwater systems (Johnson et al., 2008). However, in general, only limited data exists on the ecotoxicity of both cytotoxic and cytostatic drugs. Where ecotoxicity data do exist, 5-fluorouracil has been found to be of most concern, with EC_{50} values $\ll 1$ mg/L for a number of ecotoxicity tests.

For example, the highest toxicity was found for the bacteria, *Pseudomonas putida* (gram negative bacteria), with EC_{50} values of 0.027 mg/L and 0.044 mg/L for respective studies (Radka Zounková et al., 2009; Zounkova et al., 2010) and *Vibrio fischeri* (gram positive bacteria) with an EC_{50} value of 0.122 mg/L (Backhaus and Grimme 1999). 5-Fluorouracil was also found to have a relatively low EC_{50} value of 0.11 mg/L for *Pseudokirchneriella subcapitata*, a species of algae (Radka Zounková et al., 2009), although a markedly higher EC_{50} value of 48 mg/L was reported for another strain (*Desmodesmus subspicatus*) (Zounkova et al., 2010).

QSAR derived EC_{50} values categorise cyclophosphamide and ifosfamide as harmful for algae with a corresponding EC_{50} value of 11 mg/L for each. The QSAR data appear to be consistent with experimental data for cyclophosphamide where ecotoxicity tests for daphnia result in EC_{50} values above 100 mg/L. QSAR data for ifosfamide with zebrafish (*Brachydanio rerio*) also predict an EC_{50} value >100 mg/L and therefore was considered as low risk for this organism (Sanderson et al., 2003). For other anticancer drugs such as cisplatin, paclitaxel and gemcitabine then ecotoxicity tests with the water flea (*Daphnia magna*) have derived relatively low EC_{50} values of 0.64 mg/L, >0.74 mg/L and 1.0 mg/L, respectively (Radka Zounková et al., 2009; Zounkova et al., 2010). In addition, a relatively low EC_{50} value of 0.015 mg/L was observed for methotrexate for the African clawed frog (*Xenopus laevis*) (Bantle et al., 1996) with similarly low EC_{50}s for fish (*B. rerio*) and ciliates (protozoa) (*Tetrahymena pyriformis*) (Henschel et al., 1997). Several experimental studies have now investigated the genotoxicity of hospital effluents as an indirect marker of cytotoxic/cytostatic contamination (Giuliani et al., 1996; Buerge et al., 2006; Ferk et al., 2009; Radka Zounková et al., 2009; Zounkova et al., 2010). For example, cytarabine and gemcitabine showed genotoxicity in the umuC biological assay (based on the ability of inducing the expression of the umu operon) in both variants (with and without metabolic activation), however, the effects were observed at relatively high exposure concentrations (>100 mg/L) [51]. Cyclophosphamide and ifosfamide are also known to be present in hospital wastewater, but no independent umuC tests have been carried out to our knowledge. Genotoxicity was shown in about 13% of the hospital wastewater samples but hospital wastewater is generally diluted by at least 100-fold before entering municipal STPs (Giuliani et al., 1996). No significant genotoxicity was observed for the influent to an STP either due to dilution or degradation (Giuliani et al., 1996). In general, the umuC-test for genotoxicity appears to be less sensitive to the studied drugs than other acute and chronic ecotoxicity assays. Results from another study showed that the hospital wastewater from the oncology ward caused DNA damage in single cell gel electrophoresis (SCGE) assays in primary rat hepatocytes (Ferk et al., 2009). Cisplatin, carboplatin and 5-fluorouracil also contributed to the genotoxicity of the assay, however, they do not account for all the effects seen within the water samples (Ferk et al., 2009).

The effective toxic concentrations of anticancer drugs are generally higher than their expected and/or observed environmental concentrations (see next section). However, a maximum concentration of 124 μg/L for 5-fluorouracil was reported in hospital effluent in Austria (Mahnik, Lenz et al. 2007) and is close to some of the reported EC_{50} values reported above. These standardised chronic assays only represent exposure to one generation of organisms and prolonged multi-generational exposure might result in a number of unexpected effects, especially for compounds designed to act on DNA (Zounkova et al., 2010). Other issues that will require further research are the impact of mixtures of anti-cancer drugs as well as the biological effects arising due to exposure to metabolites or transformation products. Several of the chemicals which are likely to be present in surface waters based on observations, their use data and lack of removal in STPs also demonstrate toxicity. For example, 5-FU, gemcitabine and methotrexate have EC_{50} values $\ll 1$ mg/L for a number of different organisms that typically show less sensitivity to cyclophosphamide. Although measurements are sparse, concentrations of these chemicals in river and estuarine waters are likely to be in sub-low ng/L range (see Table 2) and therefore the risk of harm due to exposure at this concentration is likely to be minimal. Subtle effects associated with low dose exposure are an issue which requires further attention, particularly for anti-cancer drugs with high cytotoxic potency. Biochemical alterations in MCF-7 cells were observed at very low concentrations of cyclophosphamide (10^{-12} to 10^{-6} M) and representative of concentrations observed in surface waters (Strong et al., 2012).

3.6. Measured concentrations of anticancer drugs in wastewater and surface waters

Occurrence of anticancer drugs in hospital effluents has been reported in a growing number of studies, with several studies examining cytotoxic/cytostatic agents in the influent and effluent of STPs and a few studies measuring anticancer drugs in surface waters (Table 2). Most studies focus on hospital wastewaters where no treatment to the sewage is carried out (Aherne et al., 1985; StegerHartmann et al., 1996; Kümmerer et al., 1997; Steger-Hartmann et al., 1997; Mahnik et al., 2004, 2006, 2007; Moldovan 2006; Kovalova et al., 2009; Yin et al.,

Table 2
Measured concentrations of anticancer drugs in wastewaters and surface waters.

Drug name	Concentration ng/L			
	Hospital effluent	STP influent	STP effluent	Surface waters
Cyclophosphamide	<2–4500 [a, b, c, d]	<6–143 [d, e, f]	<6–< 20 [d, e, g, h]	2.2–10.1 [e, i, j]
Ifosfamide	<2–10,647 [a, c, k, l]	<0.3–13,100 [e, k, l, m, n]	1.2–2900 [e, h, k, m]	<0.5–41 [e, j]
Gemcitabine	<0.9–38 [o]	9.3 [m]	7.0 [m]	2.4 [m]
Cytarabine		9.2 [m]	14 [m]	13 [m]
Bleomycin			11–19 [p, q]	<5–17 [p, q]
Tamoxifen[a]			42–740 [r, s, t]	25 [s]
Methotrexate	<2–4689 [a, u]	59 [f]	12.6 [g]	
Doxorubicin	100–1350 [v, w]	4.5 [m]		
Vinorelbine		9.1 [m]		
5-Fluorouracil	<5–124,000 [o, w, x]			
Epirubicin	100–1400 [v]			

[a] Yin et al. (2010); [b] Moldovan (2006); [c] StegerHartmann et al. (1996); [d] Steger-Hartmann et al. (1997); [e] Buerge et al. (2006); [f] Garcia-Ac et al. (2009); [g] Castiglioni et al. (2005); [h] Ternes (1998); [i] Zuccato et al. (2000); [j] Valcárcel et al. (2011); [k] Kümmerer et al. (1997); [l] Gómez-Canela et al. (2012); [m] Martín et al. (2011); [n] Thomas et al. (2007); [o] Kovalova et al. (2009); [p] Aherne et al. (1990); [q] Halling-Sørensen et al. (1998); [r] Ashton et al. (2004); [s] Coetsier et al. (2009); [t] Roberts and Thomas (2006); [u] Aherne et al. (1985); [v] Mahnik et al. (2006); [w] Mahnik et al. (2007); [x] Mahnik et al. (2004).

[a] Tamoxifen is used for the treatment of oestrogen receptor positive breast cancers and currently licensed as an 'endocrine therapy agent' under L02BA01 of the ATC (rather than as a cytotoxic agent) and is hence not further discussed in this study. Tamoxifen is a drug with high consumption that is usually dispensed by general practitioners (i.e. 'high street' pharmacies) rather than hospital pharmacies.

Table 3
Consumption and predicted fate of anticancer drugs likely to be present in sewage effluent based on 2010–2012 consumption in NW England. Values assume urinary excretion of the unchanged drug based in Table S2 (Supplementary data) and 'best values' for estimated removal rates in STPs. For the full list of anticancer drugs see Table S5 (Supplementary data).

ATC drug	Consumption (kg/year)[a]	Consumption (μg/capita/d)[b]	Excretion of original drug %[c]	Influent load (μg/capita/d)	% of intact drug after STP biodegradation[d]	Load after STP biodegradation (μg/capita/d)	Predicted effluent conc. (ng/L)[e]	Predicted river water conc. (ng/L)[f]	Key points
L01AA01 cyclophosphamide	77.51	40	21	8.3	98.1[g]	8.2	40.9	4.1	—Continuous diffusive discharge[h] —Persistence in the environment confirmed (hospital effluents, STP wastewaters and surface waters)
L01BC06 capecitabine	357.00	183	3	5.4	85.0	4.6	23.1	2.3	—Pro-drug of 5-FU (may contribute to 5-FU load) —Continuous diffusive discharge[h] —No biodegradation studies: used as predicted biodegradation rate similar to 5-FU (85% loss)—at present this is highly uncertain.
L01BC02 fluorouracil	22.99	12	18	2.1	85.0	1.8	8.9	0.9	—No evidence of abiotic degradation is apparent. —5-FU has only been detected in hospital effluents, presence in surface waters needs confirming.
L01XX05 hydroxyurea	64.00	33	58	18.8	5.0	0.9	4.7	0.5	—By far the most consumed anticancer drug[i] —No biodegradation studies: model predictions possibly underestimate its loss, particularly when incubated with activated sludge. —Due to hydrolysis the environmental persistence of this chemical is likely to be low relative to other L01 drugs. —Presence in the environment needs confirming
L01XE01 imatinib	20.40	10	9	0.9	98.2[g]	0.9	4.6	0.5	—Concern for contamination of soils (if sludge is dispersed onto fields) and water phase. —Presence in the environment needs confirming
L01BA01 methotrexate	1.31	1	83	0.6	90.0	0.5	2.5	0.2	—Point discharge (primarily used to treat inpatients and administered 7 days/week). Diffusive discharge (outpatient clinics) —Consumption underestimated[i] —Methotrexate not marked for environmental concern in other studies [a] (removal rate of 95%), however, only 10% was removed in the first four days akin to incubation time at STPs —Confirmed detection in sewage effluent at 12.9 ng/L

L01XA02 carboplatin	5.23	3	54	1.4	30.0	0.4	2.2	0.2	—No other biodegradation studies are available for carboplatin and this assessment is based on the results from a pilot membrane bioreactor system [b].
L01BC05 gemcitabine	12.97	7	8	0.5	70.0	0.4	1.8	0.2	—Persistence in the environment confirmed (hospital effluents, STP wastewaters and surface waters)
L01CB01 etoposide	1.23	1	43	0.3	98.1[g]	0.3	1.3	0.1	—No biodegradation data —Persistence in the environment confirmed (hospital effluents and STP wastewaters)
L01AA06 ifosfamide	1.27	1	26	0.2	98.1[g]	0.2	0.8	0.1	—Point discharge (used to treat inpatients and administered 7 days/week). Continuous diffusive discharge[h] —Persistence in the environment confirmed (hospital effluents and STP wastewaters and surface waters)
L01AX04 dacarbazine	0.75	0	36	0.1	98.2[g]	0.1	0.7	0.1	—58% increased consumption from 2004 to 2008 [a]. —Presence in the environment needs confirming
L01AB02 treosulfan	1.56	1	22	0.2	70.0	0.1	0.6	0.1	—Administered at high doses (1–5 g) —Expect periodic detection or detection near hospitals that utilise this specialist chemotherapy.

[a] Besse et al. (2012).
[b] Lenz et al. (2007).
[a] Consumption total of NW England survey.
[b] Based on NW population of 5,346,000 from the populations each hospital serves.
[c] Mean excretion rate taken from clinical studies.
[d] Estimated from EPISUITE biowin model or from biodegradation data in Table S4 (Supplementary data). Predictions based on literature values are shown with an asterisk. Where no EPISUITE prediction or literature value could be obtained it was presumed that 100% of the drug remained intact.
[e] 200 L/head dilution expected in STP (Johnson et al., 2008).
[f] Further 10-fold dilution in the river (Johnson et al., 2008).
[g] Predicted from EPISUITE
[h] Communication with Blackpool Victoria hospital confirmed that treatments are more likely to commence during outpatient clinics (Mon–Fri), however, predominantly consumed by oral ingestion within the patients' own home.
[i] Used in another ATC class.

2010). Other matrices such as effluent wastewater (Aherne et al., 1990; Kümmerer et al., 1997; Steger-Hartmann et al., 1997; Halling-Sørensen et al., 1998; Ternes 1998; Castiglioni et al., 2005; Buerge et al., 2006; Martín et al., 2011) and surface waters (Valcárcel et al., 2011; Aherne et al., 1990; Halling-Sørensen et al., 1998; Zuccato et al., 2000; Buerge et al., 2006; Martín et al., 2011) have perhaps received less attention. Based on use patterns for hospitals, capecitabine, 5-fluorouracil and hydroxyurea are expected to be the most abundant chemicals present in hospital wastewater; however to date, there are only a handful of studies that have detected 5-fluorouracil in the environment and it has only been screened for in hospital wastewater (Mahnik et al., 2004, 2007; Kovalova et al., 2009). To our knowledge, capecitabine and hydroxyurea have not been screened at all, although capecitabine may contribute to the overall abundance of 5-fluorouracil detected in hospital effluent.

The two most studied cytotoxics in the environment are cyclophosphamide and ifosfamide, both of which have high consumption rates and have been found in surface waters. Based on hospital consumption, gemcitabine is expected to be an abundant chemical in the environment, however, only two studies have reported this chemical with a concentration range of <0.9–38 ng/L, similar to etoposide 3.4–380 ng/L. Methotrexate, epirubicin and doxorubicin have reported maximum concentrations in the hospital effluent of 4689, 1400 and 1350 ng/L respectively, however, they have not been detected in surface waters to our knowledge (Mahnik et al., 2006, 2007; Yin et al., 2010). A complete validity of the model is difficult due to limited measured environmental concentrations; however CP shows predicted river water concentrations within the measured environmental ranges (2.2–10 ng/L).

3.7. Priority drugs for environmental screening programmes

Table S5 (Supplementary data) reports in full the predicted environmental concentrations of the anticancer drugs in STP effluents and their surface waters based on the hospital usage data (from NW England), populations they serve, mean excretion rate and their potential for (bio)degradation during the sewage treatment processes. Based on this assessment, combined with their physical–chemical properties (e.g. dictating their partitioning behaviour) then the anticancer drugs listed in Tables 3 and 4 are considered as priority contaminants with respect to their occurrence in the wider environment and could be included in future screening programmes. Furthermore, of the 15 priority chemicals identified in this study, some of them had already been selected by other prioritisation methods (Besse et al., 2012). Other compounds such as mitotane, sorafenib, sunitinib and lapatinib, while persistent, are not expected to be present in the dissolved phase of the effluent, instead we predict that they will partition strongly to particle matter and hence be retained in sludge with the possibility of their occurrence in soil following dispersion of sewage sludge to farmland (Table 4).

The monoclonal antibodies rituximab, herceptin, cetuximab and bevacizumab may also be present in sewage treatment effluents although these are protein-based antibodies and are likely to be susceptible to biodegradation. However, there is a lack of research on their environmental fate and impact, especially for herceptin which is a widely used drug for breast cancers.

4. Conclusions

There are a wide range of anticancer drugs used in chemotherapy with consumption on a regional basis ranging from <1 to 10s kg/year. For those drugs which are only partially metabolised then wastewater and release from STPs are the major route of entry into the wider environment. From the hospitals surveyed in this study many of the anticancer drugs are administered in outpatient clinics ensuring that release of anticancer drugs is diffusive across an urban catchment and not

Table 4
Consumption and predicted fate of anticancer drugs likely to be present in sewage sludge based on 2010–2012 consumption in NW England. Values assume faecal excretion of the unchanged drug based on literature to estimate the environmental load to sludge. For the full list of anticancer drugs see Table S5 (Supplementary data).

ATC drug	Consumption (kg/year)[a]	Consumption (μg/capita/d)[b]	Faecal excretion of original drug %	Influent load (μg/capita/d)	% of intact drug after STP biodegradation[c]	Load after STP biodegradation (μg/capita/d)	K_{oc}	Key points
L01BC06 capecitabine	357.00	183	3	5.5	85	4.7	8[d]	–Partial sorption to activated sludge[e]
L01XE01 imatinib	20.40	10	20	2.1	98.2[d]	2.1	16[d]	–6% total sorption to sludge[d]
L01XE05 sorafenib	10.28	5	50[f]	2.6	49.9[d]	1.1	2884[d]	–84% total sorption to sludge[d]
L01XE07 lapatinib	4.6	2	92	2.2	33.1[d]	0.7	426,580[d]	–92% of lapatinib seen in faecal excretion –High bioaccumulation potential (K_{oc} > 5)
L01XX23 mitotane	4.5	2	60	1.4	7.4[d]	0.1	154,882[d]	–116% increased consumption from 2004 to 2008[f] –High dose (2–10 g/day) only used at specialist hospitals –High bioaccumulation potential (K_{oc} > 5)

[a] Consumption total of NW England survey.
[b] Based on NW population of 5,346,000 from the populations each hospital serves.
[c] Estimated from EPISUITE biowin model or from biodegradation data in Table S4 (Supplementary data). Predictions based on literature values are shown with an asterisk. Where no EPISUITE prediction or literature value could be obtained it was presumed that 100% of the drug remained intact.
[d] Predicted from EPISUITE.
[e] MSDS (Material Safety Data Sheet) capecitabine http://www.gene.com/download/pdf/MSDS_XELODA_Tab500mg.pdf
[f] Besse et al. (2012).

restricted to point-release with waste effluents from individual hospitals. Consumption data combined with rates of human metabolism provide an assessment of the likelihood of occurrence of these drugs in wastewater streams and hence their presence in STPs. Compiling physical–chemical property data, particularly K_{ow} and corresponding K_{oc} values, combined with degradation behaviour allows the prioritisation of a small number of chemicals. These may be of environmental concern, in that they are present in final effluent and hence can enter surface waters or are likely to persist in sewage sludge. Of the 65 anticancer drugs in use, approximately twelve drugs are recognised here as being sufficiently persistent to warrant inclusion in environmental screening programmes. Concentrations measured in surface waters for these chemicals are generally well below the EC_{50} values reported for a range of aquatic organisms. However, we recommend that further work be conducted in this area, particularly low dose 'mixture' effects, as well as understanding the environmental persistence and fate of key metabolites and transformation products.

Conflict of interest

I certify that there is no conflict of interest with any organisation regarding the material discussed in this manuscript.

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Appendix A. Supplementary data

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Supplementary Tables and Figures

Table S1: Use/consumption data of anticancer drugs in hospitals in NW England as well as selected studies

Drug name	Consumption for NW England hospitals shown in g/year and (µg/capita/d)						Average (range) for NW hospitals (g/yr)	International studies	
	University hospitals of Morecombe Bay[2]	Lancashire teaching hospitals[3]	Blackpool teaching hospitals NHS foundation[4]	East Lancashire teaching hospitals[5]	Greater Manchester & Cheshire cancer network[6]	Clatterbridge centre for oncology[7]		France, 2008 [14] (kg/yr)	University Hospital, Geneva Switzerland [64] (g/yr)1
Cyclophosphamide	1300 (9.8)	610 (4.3)	1900 (15.8)	1300 (7.1)	1400 (2.6)	71000 (84.5)	12918(310-71000)	310	610
Chlorambucil	12 (0.1)	-	10 (0.1)	-	12 (0.0)	-	6 (10-12)	8	-
Melphalan	1 (0.0)	-	20 (0.2)	-	1 (0.0)	-	4 (1-20)	5	-
Ifosfamide	150 (1.1)	-	-	-	120 (0.2)	1000 (1.2)	423 (120-1000)	100	450
Bendamustine	-	-	1 (0.0)	-	91 (0.2)	-	15 (1-91)	-	-
Busulfan	<1	-	30 (0.2)	-	-	-	5 (<1-30)	-	-
Treosulfan	200 (1.5)	860 (6.0)	400 (3.3)	100 (0.5)	-	-	260 (100-860)	-	-
Carmustine	-	-	6 (0.0)	-	-	-	1 (6)	2	-
Lomustine	-	-	6 (0.0)	-	-	42 (0.0)	8 (6-42)	3	-
Temozolomide	-	120 (0.8)	-	-	-	900 (1.1)	170 (120-900)	54	-
Dacarbazine	83 (0.6)	36 (0.3)	64 (0.5)	60 (0.3)	50 (0.1)	460 (0.5)	126 (36-460)	29	-
Methotrexate	12 (0.1)	-	130 (1.1)	7 (0.0)	260 (0.5)	900 (1.1)	218 (7-	75	410

							900)		
Pemetrexed	160 (1.2)	150 (1.1)	130 (1.1)	140 (0.8)	35 (0.1)	870 (1.0)	248 (35-870)	37	-
Mercaptopurine	16 (0.1)	-	26 (0.2)	-	90 (0.2)	-	22 (16-90)	95	-
Tioguanine	-	-	-	-	2 (0.0)	-	<1 (2)	2	-
Cladribine	<1	1 (0.0)	<1	-	<1	-	<1 (<1-1)	-	-
Fludarabine	11 (0.1)	-	9 (0.1)	-	16 (0.0)	-	6 (9-16)	6	6
Cytarabine	8 (0.1)	-	940 (7.8)	64 (0.4)	970 (1.8)	-	330 (8-970)	130	670
5-Fluorouracil	2500 (18.9)	3100 (21.8)	1800 (14.9)	790 (4.3)	1800 (3.4)	13000 (1.5)	3832 (790-13000)	1700	3100
Tegafur	-	58 (0.4)	-	-	-	58 (0.0)	19 (58)	37	-
Gemcitabine	1300 (9.8)	2300 (16.1)	860 (7.1)	1600 (8.8)	110 (0.2)	6800 (0.8)	2162 (110-6800)	380	660
Capecitabine	29000 (218.8)	64000 (449.3)	34000 (282.1)	-	-	230000 (27.4)	59500 (29000-230000)	5100	-
Azacitidine	18 (0.1)	-	11 (0.1)	28 (0.2)	17 (0.0)	-	12 (11-28)	-	-
Vinblastine	2 (0.0)	-	2 (0.0)	<1	1 (0.0)	-	1 (<1-2)	1	-
Vincristine	1 (0.0)	1 (0.0)	1 (0.0)	<1	1 (0.0)	<1	1 (<1-1)	<1	1
Vinorelbine	13 (0.1)	19 (0.1)	45 (0.4)	-	1 (0.0)	220 (0.0)	50 (1-220)	13	-
Etoposide	190 (1.4)	90 (0.6)	210 (1.7)	3 (0.0)	38 (0.1)	700 (0.1)	205 (3-700)	41	110
Paclitaxel	35 (0.3)	200 (1.4)	39 (0.3)	57 (0.3)	140 (0.3)	390 (0.0)	144 (35-390)	39	79
Docetaxel	58 (0.4)	74 (0.5)	44 (0.4)	53 (0.3)	43 (0.1)	260 (0.0)	89 (44-260)	27	43
Trabectedin	-	-	-	-	-	6 (0.0)	1 (6)	-	-
Dactinomycin	-	-	-	-	-	72 (0.0)	12 (72)	-	-

Doxorubicin	26 (0.2)	6 (0.0)	14 (0.1)	27 (0.1)	32 (0.1)	88 (0.0)	32 (6-88)	17	30
Daunorubicin	-	-	15 (0.1)	-	11 (0.0)	-	4 (11-15)	1	-
Epirubicin	87 (0.7)	95 (0.7)	70 (0.6)	96 (0.5)	61 (0.1)	44 (0.0)	76 (44-96)	18	41
Idarubicin	<1	-	1 (0.0)	-	1 (0.0)	-	<1 (<1-1)	<1	-
Mitoxantrone	<1	-	1 (0.0)	-	<1	-	<1 (<1-1)	<1	-
Bleomycin	1 (0.0)	4 (0.0)	2 (0.0)	<1	2 (0.0)	7 (0.0)	3 (<1 -4)	1	-
Mitomycin C	16 (0.1)	<1	11 (0.1)	13 (0.1)	3 (0.0)	<1	8 (<1-16)	3	9
Cisplatin	45 (0.3)	190 (1.3)	39 (0.3)	43 (0.2)	16 (0.0)	370 (0.0)	117 (16-370)	23	67
Carboplatin	320 (2.4)	950 (6.7)	540 (4.5)	600 (3.3)	15 (0.0)	2800 (0.3)	871 (15-2800)	84	330
Oxaliplatin	96 (0.7)	130 (0.9)	110 (0.9)	89 (0.5)	74 (0.1)	510 (0.1)	168 (74-510)	33	64
Procarbazine	24 (0.2)	-	-	-	3 (0.0)	120 (0.0)	25 (3-120)	35	-
Rituximab	-	120 (0.8)	320 (2.7)	-	310 (0.6)	18 (0.0)	128 (18-320)	72	-
Trastuzumab	250 (1.9)	370 (2.6)	160 (1.3)	260 (1.4)	-	2000 (0.2)	507 (160-2000)	56	-
Alemtuzumab	-	-	-	-	30 (0.1)	-	5 (30)	-	-
Cetuximab	17 (0.1)	110 (0.8)	57 (0.5)	-	-	270 (0.0)	76 (17-270)	55	-

"-" = not recorded

[1] Calculated using the average dose (mg) from NW hospital survey

[2] Comprising of Furness general hospital, Royal Lancaster Infirmary, Westmorland general hospital, Queen Victoria hospital, Ulverston community health centre. Population served 363,000

[3] Comprising of Royal Preston hospital (Rosemere cancer foundation) and Chorley and South Ribble hospital. Population served 390,000

[4] Comprising of Blackpool Victoria hospital, Clifton hospital, Fleetwood hospital and three elderly rehabilitation hospitals. Population served 330,000

[5] Comprising of Burnley general hospital, Royal Blackburn hospital and Inpatient rehabilitation services are also provided at Pendle community hospital and the Rakehead unit at Burnley general hospital. Outpatient and diagnostic services are also provided at the Accrington Victoria, Clitheroe hospital, Rossendale and St Peters Primary health care centre's. Population served 500,000

[6] Comprising of NHS Trafford (Trafford general hospital host to the Trafford Macmillan care centre); Bolton NHS Foundation trust (Royal Bolton hospital); Salford Royal NHS Foundation trust (Salford Royal hospital); Mid Cheshire hospitals NHS foundation trust (Leighton hospital, Victoria Infirmary and Elmhurst intermediate care centre); Wrightington, Wigan and Leigh NHS foundation trust (Royal Albert Edward Infirmary, Leigh Infirmary, Wrightington hospital and Thomas Linacre centre (provides the majority of out-patient services for the Trust). Population served 1,463,000

[7] Comprising of outpatient clinics at Linda McCartney centre (Royal Liverpool University hospital), the Countess of Chester, Southport hospital, Halton general hospital, Aintree University hospital, Broadgreen hospital and The Liverpool Woman's. Population served 2,300,000

Table S2: Average urinary excretion rates of the unchanged parent drug

% of administered drug excreted (i.e. not metabolized)					
<5%	5-15%	15-25%	25-45%	45-75%	>75%
Chlorambucil[1, 2, 3, 4] [1] Busulfan[1, 2, 5] [2] Lomustine[1, 2] Capecitabine[1, 2] [3] Trabectedin[1, 2] [4] Idarubicin[2] [5] Gefitinib[1, 2] Sorafenib[1, 2] Dasatinib[1] Laptinib[1] [6] Nilotinib[2, 5] Temsirolimus[1, 2] Everolimus[1, 2] Pazopanib[1, 2] Bortezomib*** Trastuzumab*** Alemtuzumab*** Rituximab*** Cetuximab*** Bevacizumab***	Temozolomide[1, 2] [7, 8] Mercaptopurine[1, 2] Cytarabine[2] Gemcitabine[1] [9] Vinblastine[2] [10] Vincristine[2,] [11] Vinorelbine[1, 2] Paclitaxel[1, 2] Docetaxel[1, 2] Doxorubicin[1, 2, 3] [12] Epirubicin[1, 2] [12] Mitoxantrone[1, 2] Mitomycin[1, 2] Procarbazine[2, 4] Imatinib[1, 2] Erlotinib[1, 2] Mitotane[1, 2, 4, 6] [13] Eribulin[1]	Cyclophosphamide[1, 2, 3] [12, 14-17] Melphalan[1, 2] [18] Bendamustine[1] Treosulfan [19-22] Tioguanine [23, 24] 5-Fluorouracil[1, 2] [3, 9, 12] Tegafur[1] Dactinomycin[1] Sunitinib[1, 2] [25] Irinotecan[1, 2]	Ifosfamide[2, 3] [17, 26, 27] Dacarbazine[1, 2] Cladribine[1, 2] Fludarabine[1, 2] Etoposide[1, 2, 3] Daunorubicin[1, 2] [28] Cisplatin[1] [8] Oxaliplatin[1, 2] [8] Topotecan[1, 2]	Carmustine[1, 2] Azacitidine[2] Bleomycin[1, 2] Carboplatin[1, 2] [8] Hydroxyurea[1, 2] Tretinoin[1, 2]	Methotrexate[2, 3] [29] Pemetrexed[1, 2, 3] [30]

[1] electronic Medicines Compendium (eMC) (http://www.medicines.org.uk/EMC)
[2] Product monograph (http://www.bccancer.bc.ca/HPI/DrugDatabase/DrugIndexPro/default.htm)
[3] RxList (http://www.rxlist.com/script/main/hp.asp)
[4] Drugs.com (http://www.drugs.com/)
[5] Ema (http://www.ema.europa.eu/ema/)
[6] ToxNet (http://toxnet.nlm.nih.gov/)
*** No available data (Urinary excretion data was not available for six L01X anticancer drugs)

Table S3: Physiochemical properties of selected cytotoxic drugs and their classification according to the Anatomical Therapeutic Classification (ATC) system.

ATC	Drug name	pKa	Charge at pH 7.4	Weak acid/weak base	Log Kow	Log Dow at pH 7.4	Koc[4]	BCF[4]	Solubility in water (mg/L) at 25°C[4]
L01AA01	Cyclophosphamide	2.84, 6.00 [31, 32]	Neutral	Acid [32]	0.63		44	3	4.00E+04
L01BC06	Capecitabine	8.8 [33]	Neutral	Acid	0.96*		8	3	8.23E+02
L01BC02	5-Fluorouracil	7.6-8.0, 13.0 [32-34]	Neutral	Acid [32]	-0.93		4	3	1.11E+04
L01XX05	Hydroxyurea	10.6*	Neutral	Acid	-1.27		3	3	7.91E+04
L01XE01	Imatinib	8.07, 3.73, 2.56, 1.52*	Positive	Base		0.19	16	3	6.48E+01
L01BA01	Methotrexate	3.80, 4.8, 5.6 [33]	Negative	Acid		-1.41	20	3	4.98E+03
L01XA02	Carboplatin	0.24, 3.55*		Base	-1.78	0.01	891 [35]		
L01BC05	Gemcitabine	3.6 [34]	Neutral	Base	-1.24		1	3	1.53E+04[6]
L01CB01	Etoposide	9.8 [36]	Neutral	Acid	0.60		19	3	5.87E+01
L01AA05	Ifosfamide	1.45-4.0 [31, 32]	Neutral	Base [32]	0.86		51	3	3.78E+03
L01AX04	Dacarbazine	4.42*	Neutral	Base	-0.24		15	10	4.22E+03
L01AB02	Treosulfan	12.36*	Neutral	Acid	-2.09*		1	3	7.00E+04[8]
L01XX23	Mitotane	N/A	Neutral	N/A	6.11*		154882	4989	1.00E-01
L01XE07	Lapatinib	3.80, 7.20[2]*	Positive	Base		4.72	426580	1127	9.06E-02
L01CD01	Paclitaxel	11.99	Neutral	Zwitterion [32]	5.25		58884	750	1.07E-04
L01AA03	Melphalan	1.83, 9.13 [37]	Neutral	Acid [38]	-0.52		14	3	2.71E+02
L01AA09	Bendamustine	0.88, 4.17, 6.94[5]	Negative	Zwitterion/Acid		2.84	977	3	2.69E+01
L01AD01	Carmustine	12.27*	Neutral	Acid	1.53		89	5	1.83E+03
L01AX03	Temozolomide	N/A [39]	Neutral	Base	1.15		29	3	1.81E+03
L01BA04	Pemetrexed	3.6, 4.4 [33]	Negative	Acid		-2.43	60	3	1.84E+02

L01BB02	Mercaptopurine	7.9 [40]	Neutral	Acid [40]	0.67 [40]		40	3	1.98E+04
L01BB05	Fludarabine	3.2, 5.8*	Negative	Acid		-1.22	2	3	1.44E+04
L01BC01	Cytarabine	4.2 [34]	Neutral	Base	-2.15		1	3	8.66E+04
L01BC03	Tegafur	7.98[1]	Neutral	Acid	-0.27		6	3	3.64E+03
L01BC07	Azacitidine	2-3 [41]	Neutral	Base	-2.17		1	3	8.89E+04
L01CA04	Vinorelbine	7.4, 5.4 [42, 43]	Positive	Base	4.72*	4.57	30200	604	9.86E-03
L01CD02	Docetaxel	12.02*	Neutral	Acid	3.64*		27	65	5.17E-03
L01DA01	Dactinomycin	8.06 [44]	Neutral	Acid	1.42*				
L01DB01	Doxorubicin	7.34, 8.3, 9.46 [31, 33, 45]	Positive	Base [32]		-1.93	389	3	5.34E+02
L01DB02	Daunorubicin	8.4 [31, 45]	Positive	Base [32]		-0.14	490	1	1.23E+02
L01DB03	Epirubicin	7.7 [31]	Positive	Base*		-0.30	372	4	6.25E+02
L01DC01	Bleomycin	7.3 [46]	Positive	Base		-0.47			
L01DC03	Mitomycin C	3.2	Neutral	Base	-0.38		76	3	8.11E+03
L01XA01	Cisplatin	6.6, 5.5, 7.3 [47]			-2.40	-2.19	12589 [35]		
L01XA03	Oxaliplatin	7.35, 9.99*		Base	-1.63	-1.42			
L01XB01	Procarbazine	6.8*	Neutral	Base	-0.82*		18	3	8.32E+03
L01XE03	Erlotinib	5.42*	Neutral	Base	2.96*		2188	42	9.97E+00
L01XE04	Sunitinib	8.95*	Positive	Base		0.76	891	29	1.52E+01
L01XE05	Sorafenib	11.55, 2.03[2]*	Neutral	Base/Acid	4.39*		2884	366	2.14E-01
L01XE08	Nilotinib	2.1, 5.4[1]	Neutral	Base	3.60		79433	110	3.90E-01
L01XE11	Pazopanib	2.1, 6.4, 10.2[3]	Neutral	Base/Acid	3.38*		12023	79	2.32E+00
L01XX14	Tretinoin	5.0*	Negative	Acid		4.30			
L01XX17	Topotecan	0.60, 6.99, 10.50 [48]	Positive	Base/Acid		-3.13	107	3	3.30E+02
L01XX19	Irinotecan	8.1*	Positive	Base		3.24	2818	355	3.64E-02
L01XX32	Bortezomib	13.82*	Neutral	Acid	1.47*		766	4	2.13E+02
L01XX41	Eribulin	9.59[2]	Positive	Base		-0.34	1288	14	2.70E+00

[1] www.ema.europa.eu/docs/en_GB/.../WC500034398.pdf
[2] *ACD properties calculator (*http://www.chemicalize.org/structure*)*
[3] http://www.medicines.org.au/files/gwpvotri.pdf
[4] BCF predicted with EPI SUITE: linear relationship with kow does not hold for many compounds with high polarity (see text)
[5] http://www.faqs.org/patents/app/20090264488
[6] MSDS gemcitabine (http://ehs.lilly.com/msds/Gemzar.pdf)
[7] MSDS imatinib (http://export.fass.se/pdfprint/servlet/se.itsip.pdfprint.servlets.ConvertServlet?nplId=20031111000058&docTypeId=78&userType=2&paraImported=null&orgNplId=null&showParaLink=null&hasEnvSection=yes&paraInfo=null&orgCompany=null&docId=ID18IILYT1XUZ1XGCS_IDX0000000180&fontSize=standard)
[8] MSDS treosulfan (http://www.medac.de/medac_international/data/SDS/treosulfan_E.pdf)
*From predicted data
** Calculated as an average, with consideration to dastinib a similar compound to basic ionization

Table S4: Degradative loss processes: biodegradation and hydrolysis

Drug Name	Biodegradation				Hydrolysis			% STP total removal (EPI suite)	Ref.
	Test	Incubation (days)	Initial conc.	Results	Log k_{OH} (L/mole.s)[1]	% loss in 5days			
						at pH 7.0	at pH 8.1		
Cyclophosphamide	ZWT[2] (OECD 302B)	28	51.7mg/L	No degradation	-8.458	Neg.	Neg.	1.86	[49]
	OECD confirmatory	10	375, 750mg/L	0±5% degradation					[50]
	CBT[3] (OECD 1992)	40	4.3mg/L	28-66% degradation in 40 days. Chemical structurally related to CP					[51]
	ZWT[2] (OECD 1992)	40	200mg/L	5-72% degradation in 28 days. Chemical structurally related to CP					[51]
	AS incubation	1	90, 900ng/L	No degradation					[52]
Ifosfamide	ZWT[2] (OECD 302B)	42	51.7mg/L	No degradation	-7.397	Neg.	Neg.	1.88	[53]
	STP simulation	42	11.4µg/L	Negligible					[53]

	test (OECD 1992)								
	CBT[3] (OECD 1992)	40	4.3mg/L	28-66% degradation in 40 days. Chemical structurally related to IF					[51]
	ZWT[2] (OECD 1992)	40	200mg/L	5-72% degradation in 28 days. Chemical structurally related to IF					[51]
Treosulfan	CBT[3] (OECD 301D)	40	5mg/L	30% degradation in the first 28 days (40% in 40 days)	N/A	N/A	N/A	1.85	[54]
Methotrexate	OECD confirmatory (AS incubation)	10	10, 20mg/L	98±6% degradation. > 10% in the first four days	N/A	N/A	N/A	1.85	[50]
Pemetrexed	Ready biodegradability	29	N/A	20% was released as CO^2	N/A	<10% (at 50°C)[4]	N/A	1.85	MSDS[4]
	Biodegradation (sludge)	1	N/A	>99% disappearance when incubated with 1.5g/L					MSDS[4]

				sludge solids. After 1 hour incubation 90% of pemetrexed had degraded					
Fludarabine	N/A	N/A	N/A	N/A	-4.097	Neg.	Neg.	1.85	N/A
Cytarabine	OECD confirmatory AS incubation	10	12.5, 25mg/L	60±8% degradation, 2 days = ~10%	N/A	N/A	N/A	1.85	[50]
	CBT[3] (OECD 301D)	40	N/A	50% degradation after 20days					[55]
	ZWT[2] (OECD 302B)	40	N/A	>95% degradation HOW MANY DAYS?					[55]
5-fluorouracil	CBT[3] (OECD 301D)	28 and 40	9.02mg/L	No degradation	N/A	N/A	N/A	1.85	[55]
	ZWT[2] (OECD 302B)	28	854mg/L	2% degradation					[55]
	OECD 303A confirmatory AS incubation	10	5, 10, 20mg/L	100±4% (15% after 1 day and a sharp increase to 100% on day 2) Higher degradation rate with lower concentrations					[50]
	AS incubation	50	50ug/L	<60% removal					[56]

				after 50 days					
	AS incubation	1	5, 500ug/L	Complete degradation					[57]
	ZWT[2] (0ECD 302B)	21	270mg/L	No degradation, using pre-adapted AS					[3]
	Inherent biodegradation test, 4g AS/L and closed test vessels	14	0.2, 11.4mg/L	97.5->100% degradation. > 25% Biodegradation in 1day					[3]
	OECD 303A	3	10mg/L	38-92% biodegradation					[3]
Gemcitabine	CBT[3] (OECD 301D)	40	1660mg/L	45% Degradation	N/A	Neg.[5]	N/A	1.85	[55]
	ZWT[2] (OECD 302B)	40	1660mg/L	50% degradation					[55]
	Aerobic biodegradation	28		30% degradation					MSDS[5]
Capecitabine	ZWT[2] (OECD 303B)	28	N/A	58% degradation (15% removed in 7 days)	N/A	N/A	N/A	1.85	[3]
	Like OECD 302C	21	30mg/L	41% mineralization, 27% mineralization in 14 days					[3]
	Like OECD	84		55-66%					[3]

	302C			mineralization, 29% mineralization in 28 days					
Vinblastine	CBT[3]	28	N/A	10% Degradation	0.016/-0.835/-1.261	0.63-4.48	7.95-56.40	NOT ON LIST	[58]
	ZWT[2]	40	N/A	18% degradation					[58]
Vincristine	CBT[3]	28	N/A	30% degradation	-0.788/0.149	0.70-6.09	8.86-76.60	NOT ON LIST	[58]
Vinorelbine	N/A	N/A	N/A	N/A	-0.844/0.004/-1.261	0.24-4.36	2.98-54.90	66.90	N/A
Etoposide	N/A	N/A	N/A	N/A	-2.689	0.01	0.11	1.86	N/A
Paclitaxel	N/A	N/A	N/A	N/A	-0.297/-1.669/0.000/-0.433	0.09-4.32	1.17-54.40	84.19	N/A
Docetaxel	N/A	N/A	N/A	N/A	-0.317/-1.689/-0.452/4.667	0.00-2.08	0.00-26.20	16.63	N/A
Doxorubicin	AS incubation	1	2500ug/L	48-74% degradation (20-40% recovered in sludge, 6-12% recovered in liquid phase). Degraded mainly due to adsorption to	N/A	N/A	N/A	1.85	[57]

				sludge					
Epirubicin	CBT[3] (OECD 301D)	N/A	5mg/L	No degradation	N/A	N/A	N/A	1.85	[59]
	ZWT[2] (OECD 302B)	N/A	N/A	Degraded, mainly due to adsorption to sludge					[59]
	ZWT[2] (OECD 302B) CBT[3] (OECD 301D)	N/A	N/A	Eliminated in ZWT but not in CBT					[57]
Mitoxantron	CBT[3] (OECD 301D)	40	5mg/L	No degradation	N/A	N/A	N/A		[54]
Mitomycin	N/A	N/A	N/A	N/A	-2.218	0.03	0.33	1.85	N/A
Cisplatin	OECD screening test	21	0, 0.32, 1.6mg/L	0±2% Degradation	N/A	N/A	N/A	N/A	[50]
Imatinib	Aerobic, 92/69/EC (L383) C.4-C	28	N/A	9-12%; not readily biodegradable	N/A	N/A	N/A	1.85	MSDS[6]
Topotecan	N/A	N/A	N/A	N/A	-0.986	0.45	5.62	1.85	N/A
Irinotecan	N/A	N/A	N/A	N/A	-1.127/0.000	0.32-4.32	4.06-54.40	8.33	N/A

Neg. – negligible
N/A – not available
AS – activated sludge
STP – sewage treatment plant
[1] Second order rate constant estimated using the SPARC model (http://archemcalc.com/sparc/test/login.cfm?CFID=250050&CFTOKEN=79322869)
[2] ZWT - Zahn-Wellens Test (test for inherent biodegradability – OECD302)

[3] CBT – Closed Bottle Test (OECD 301)
[4] MSDS pemetrexed (http://ehs.lilly.com/msds/Alimta.pdf)
[5] MSDS gemcitabine (http://ehs.lilly.com/msds/Gemzar.pdf)
[6] MSDS imatinib (http://export.fass.se/pdfprint/servlet/se.itsip.pdfprint.servlets.ConvertServlet?nplId=20031111000058&docTypeId=78&userType=2&paraImported=null&orgNplId=null&showParaLink=null&hasEnvSection=yes&paraInfo=null&orgCompany=null&docId=ID18IILYT1XUZ1XGCS_IDX0000000180&fontSize=standard)

Table S5: Consumption and predicted fate of anticancer drugs likely to be present in sewage effluent based on 2010-2012 consumption in NW England. Values assume excretion of the unchanged drug based on Table S2 (Supplementary information) and 'best values' for estimated removal rates in STPs.

ATC Drug	Consumption (kg/year)[1]	Consumption (µg/capita/d)[2]	Excretion of original drug %[3]	Influent load (µg/capita/d)	% of intact drug after STP biodegradation[4]	Load after STP biodegradation (µg/capita/d)	Predicted effluent conc. (ng/L)[5]	Predicted river water conc. (ng/L)[6]	Discussion
L01AA01*** Cyclophosphamide	77.51	40	21	8.4	98.1*	8.3	41.3	4.1	- Continuous diffusive discharge[7] - Persistence in the environment confirmed (hospital effluents, STP wastewaters and surface waters)
L01BC06*** Capecitabine	357.00	183	3	5.4	85.0	4.6	23.1	2.3	- Pro-drug of 5-FU (may contribute to 5-FU load) - Continuous diffusive discharge[7] - No biodegradation studies: used a predicted biodegradation rate similar to 5-FU (85% loss) – at present this is highly uncertain.
L01BC02*** Fluorouracil	22.99	12	18	2.1	85.0	1.8	8.9	0.9	- No evidence of abiotic degradation is apparent. - 5-FU has only been detected in hospital effluents, presence in surface waters needs confirming.

L01XX05*** Hydroxyurea	64.00	33	58	18.8	5.0	0.9	4.7	0.5	- By far the most consumed anticancer drug[8] - No biodegradation studies: model predictions possibly underestimate its loss, particularly when incubated with activated sludge. - Urease catalyses the hydrolysis of urea, it also catalyses the hydrolysis of HU. - Due to hydrolysis the environmental persistence of this chemical is likely to be low relative to other L01 drugs. - Presence in the environment needs confirming
L01XE01*** Imatinib	20.40	10	9	0.9	98.2*	0.9	4.6	0.5	- Concern for contamination of soils (if sludge is dispersed onto fields) and water phase. - Presence in the environment needs confirming
L01BA01*** Methotrexate	1.31	1	83	0.6	90.0	0.5	2.5	0.2	- Point discharge (Primarily used to treat inpatients and administered 7days/week). Diffusive discharge (outpatient clinics) - Consumption underestimated[8] - Methotrexate not marked for environmental concern in other studies [ref] (removal rate of 95%), however, only 10% was removed in first four days akin to incubation time at STPs - Confirmed detection in sewage effluent at 12.9ng/L
L01XA02*** Carboplatin	5.23	3	54	1.4	30.0	0.4	2.2	0.2	- No other biodegradation studies are available for carboplatin and this assessment is based on the results from a pilot membrane bioreactor system [ref].
L01BC05*** Gemcitabine	12.97	7	8	0.5	70.0	0.4	1.8	0.2	- Persistence in the environment confirmed (hospital effluents, STP wastewaters and surface waters)
L01CB01*** Etoposide	1.23	1	43	0.3	98.1*	0.3	1.3	0.1	- No biodegradation data - Persistence in the environment confirmed (hospital effluents and STP wastewaters)

L01XA03 Oxaliplatin	1.01	1	40	0.2	100.0**	0.2	1.0	0.1	
L01AA06*** Ifosfamide	1.27	1	26	0.2	98.1*	0.2	0.8	0.1	- Point discharge (used to treat inpatients and administered 7days/week). Continuous diffusive discharge[7] - Persistence in the environment confirmed (hospital effluents and STP wastewaters and surface waters)
L01AX04*** Dacarbazine	0.75	0	36	0.1	98.2*	0.1	0.7	0.1	- 58% increased consumption from 2004 to 2008 [ref]. - No biodegradation data available - Presence in the environment needs confirming
L01AB02*** Treosulfan	1.56	1	22	0.2	70.0	0.1	0.6	0.1	- Administered at high doses (1-5g) - Expect periodic detection or detection near hospitals that utilise this specialist chemotherapy.
L01XA01 Cisplatin	0.70	0	33	0.1	98.2*	0.1	0.6	0.1	
L01XE03 Erlotinib	4.14	2	6	0.1	94.6*	0.1	0.6	0.1	
L01BC01 Cytarabine	1.98	1	10	0.1	90.0	0.1	0.5	0.0	
L01BA04 Pemetrexed	1.49	1	80	0.6	10.0	0.1	0.3	0.0	
L01XE04 Sunitinib	0.70	0	16	0.1	98.1*	0.1	0.3	0.0	
L01XX19 Irinotecan	0.56	0	16	0.0	91.7*	0.0	0.2	0.0	
L01AX03 Temozolomide	1.02	1	7	0.0	98.1*	0.0	0.2	0.0	
L01BC07 Azacitidine	0.07	0	68	0.0	98.2*	0.0	0.1	0.0	

L01DB03 Epirubicin	0.45	0	11	0.0	98.2*	0.0	0.1	0.0	
L01CD02 Docetaxel	0.53	0	7	0.0	83.4*	0.0	0.1	0.0	
L01BC03 Tegafur	0.12	0	20	0.0	98.2*	0.0	0.1	0.0	
L01DB01 Doxorubicin	0.19	0	14	0.0	80.0	0.0	0.1	0.0	
L01XX23**** Mitotane	4.50	2	6	0.1	7.4*	0.0	0.1	0.0	- High dose (2-10g/day) only used at specialist hospitals - High bioaccumulation potential (log K_{oc} > 5)
L01XE07**** Lapatinib	4.60	2	1	0.0	33.1*	0.0	0.1	0.0	- 92% of lapatinib seen in fecal excretion - High bioaccumulation potential (log K_{oc} > 5) - 116% increased consumption from 2004 to 2008 [ref].
L01AA09 Bendamustine	0.09	0	20	0.0	95.5*	0.0	0.0	0.0	
L01XB01 Procarbazine	0.15	0	11	0.0	98.2*	0.0	0.0	0.0	
L01CA04 Vinorelbine	0.30	0	13	0.0	33.1*	0.0	0.0	0.0	
L01DA01 Dactinomycin	0.07	0	17	0.0	100.0**	0.0	0.0	0.0	
L01BB05 Fludarabine	0.04	0	34	0.0	98.2*	0.0	0.0	0.0	
L01XE08 Nilotinib	0.57	0	2	0.0	84.5*	0.0	0.0	0.0	
L01DC01 Bleomycin	0.02	0	62	0.0	100.0**	0.0	0.0	0.0	
L01XX17 Topotecan	0.03	0	33	0.0	98.2*	0.0	0.0	0.0	

L01CD01**** Paclitaxel	0.86	0	7	0.0	15.8*	0.0	0.0	0.0	- 61% of paclitaxel seen in fecal excretion - High bioaccumulation potential (log K_{oc} > 5)
L01XX14 Tretinoin	0.02	0	63	0.0	54.5*	0.0	0.0	0.0	
L01DB02 Daunorubicin	0.03	0	25	0.0	98.2*	0.0	0.0	0.0	
L01XX41 Eribulin	0.06	0	9	0.0	98.2*	0.0	0.0	0.0	
L01XE05 Sorafenib	10.28	5	0	0.0	49.9*	0.0	0.0	0.0	
L01XX32 Bortezomib	0.00	0	100	0.0	98.0*	0.0	0.0	0.0	
L01DC03 Mitomycin	0.04	0	10	0.0	98.2*	0.0	0.0	0.0	
L01AA03 Melphalan	0.02	0	20	0.0	98.2*	0.0	0.0	0.0	
L01AD01 Carmustine	0.01	0	57	0.0	98.0*	0.0	0.0	0.0	
L01XE11 Pazopanib	0.05	0	4	0.0	89.4*	0.0	0.0	0.0	
L01BB02 Mitoxantrone	0.00	0	7	0.0	100.0**	0.0	0.0	0.0	

[1] Consumption total of NW survey

[2] Based on NW population of 5,346,000 from the populations each hospital serves

[3] Mean excretion rate taken from *n* clinical studies

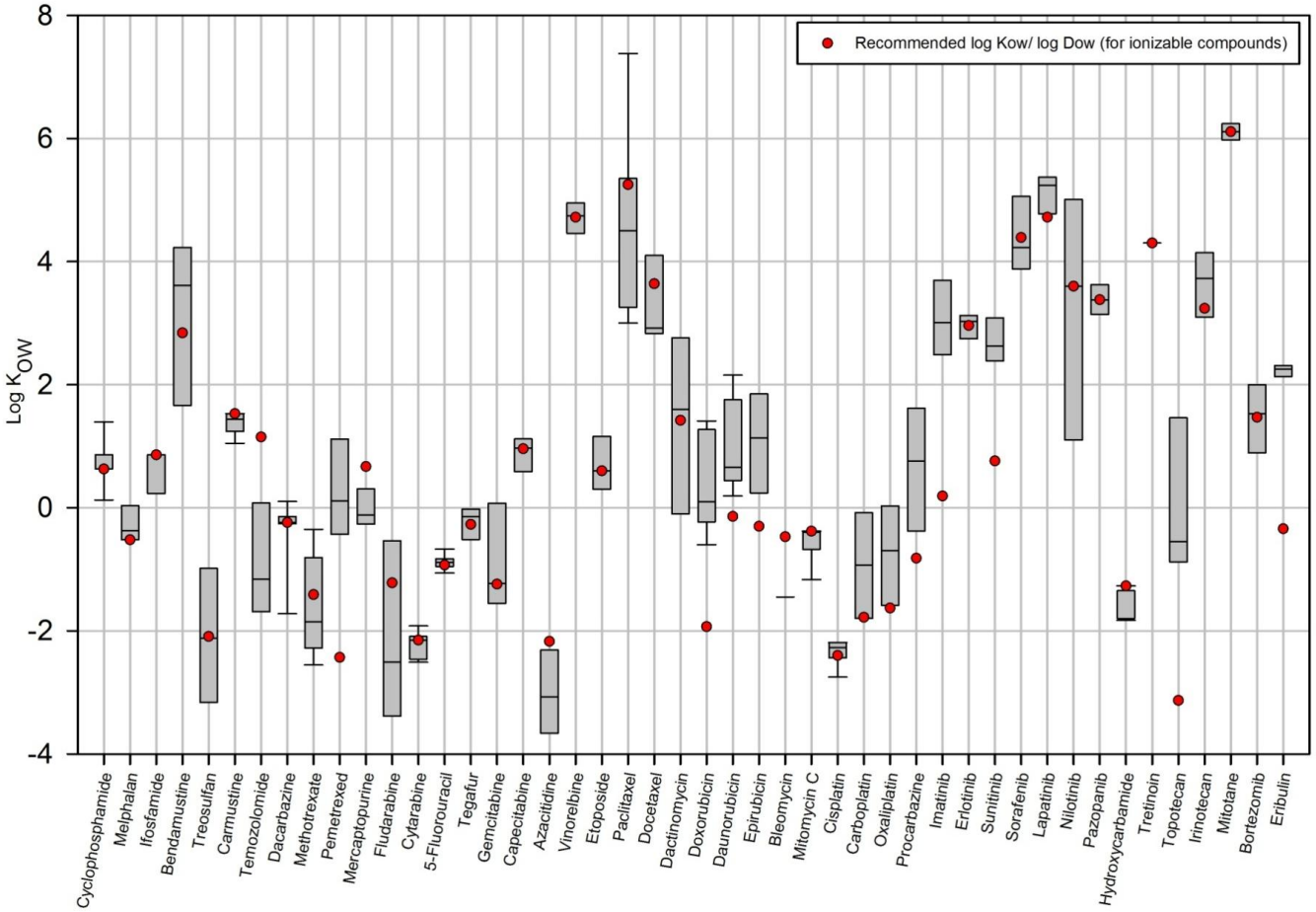
[4] Estimated from EPISUITE biowin model or from biodegradation data table 4. Predictions based on literature values are shown with an asterisk. Where no EPISUITE prediction or literature value could be obtained it was presumed that 100% of the drug remained intact.

[5] 200L/head dilution expected in STP [12]

[6] Further 10-fold dilution in the river [12]

[7] Communication with Blackpool Victoria hospital confirmed that treatments are more likely to commence during outpatients clinics (Mon-Fri), however, predominantly consumed by oral ingestion within the patients' own home)
[8] Used in another ATC class
* Predicted from EPISUITE
** No rate available
*** Priority chemical in surface water
**** Priority chemical in soil

Figure S1: Box and whisker plot of log K_{ow} values for a wide number of anticancer drugs. For each chemical the K_{ow} values were obtained from the literature (i.e. empirically observed) or calculated (*n*=1-22)



Recommended log K_{ow} based on the most reliable data sources with consideration of D_{ow} for ionisable compounds

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Paper II

A survey of two common-use anticancer drugs in WWTPs and receiving waters: chemical fate and river water loads.

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Pharmaceuticals; anticancer drugs; sewage; influent/effluent; receiving water; river

Abstract

The presence of anticancer drugs in wastewaters, effluents and surface waters are a growing environmental concern. However, environmental measurements encompassing wastewater, treated effluent and receiving water required to assess exposure from sewage treatment plants (STPs) are lacking. In this study, raw influent (*n*=16) and final effluent waters (*n*=16) from fourteen STPs were sampled across England for the presence of cyclophosphamide (CP) and ifosfamide (IF) – two widely used anticancer drugs – using a sensitive analytical method. CP was detected in both influent and treated effluent with mean (SD) concentrations of 4.1 ng/L (4.8) and 6.6 ng/L (6.5), respectively. IF was only detected in four effluent samples (mean ~ 0.38 ng/L (n=4)). Surveys along the rivers Calder, Darwen and Ribble (located in North West UK) demonstrate increasing loads of CP down the catchment. CP was present at 5 of the 6 river locations with concentrations ranging from 0.41 to 3.71 ng/L. It was noted that CP concentration actually increased during treatment implying a deconjugation process was taking place. This also confirmed its high persistence observed by previous authors.

1. Introduction

Anticancer drugs are a wide group of pharmaceuticals that are receiving increasing attention as environmental contaminants, with an increasing number of studies reporting levels in wastewater (Llewellyn et al., 2011, Buerge et al., 2006, Zuccato et al., 2000, Martín et al., 2011). There are over 70 separate chemicals classified as antineoplastic and immuno-modulating agents that are subdivided under the Anatomical Therapeutic Chemical (ATC) classification scheme into five groups of chemotherapy agents. These include the two important groups of alkylating agents (L01A) and antimetabolites (L01C) (Booker et al., 2014).

Consumption of anticancer drugs is approximately <10-100's kg/yr on a country wide basis with cyclophosphamide (CP) and ifosfamide (IF) being two commonly used alkylating agents used in the treatment of a wide range of cancers (e.g. bronchial, breast and ovarian cancers as well as certain types of leukaemia) (Booker et al., 2014). Johnson et al. (2013) reported a 15 fold difference in CP consumption across Europe ranging from 2.3μg/capita/day (Finland, 2010) to 35.7μg/capita/day (Sweden, 2010) with a mean consumption of 10.4 μg/capita/day (Johnson et al., 2013). A hospital survey conducted across NW England (2011) showed CP consumption ranged between 4.3-11.8 μg/capita/day (Booker et al., 2014). IF is a synthetic analog of CP and is consumed to a lesser extent, only 1.2 μg/capita/day is consumed in NW England (Booker et al., 2014). These drugs can be administered as a single active ingredient or in combination with other anticancer drugs to increase their efficiency, and are administered to patients residing in hospital as well as 'out-patients' living in the wider community. Metabolism for CP/IF is estimated to be ~55-85% of the administered dose, ensuring that urinary and fecal excretion and transport of

wastewater through STPs is the most significant route of entry of these chemicals into the wider aquatic environment (Booker et al., 2014, Rowney et al., 2009).

Interest in this specialised group of pharmaceuticals as environmental contaminants has increased principally due to their deliberately toxic mode of action (Johnson et al., 2008). The potential for these drugs to harm humans through water recycling in areas where river/lake water is abstracted for potable water supplies has provided a greater focus (Johnson et al., 2008). Long term exposure may cause subtle genetic alterations that may not be present in the limited number of toxicity tests carried out over one generation. Alkylating agents such as CP and IF act via a cytotoxic mode of action, CP (a prodrug) undergoes complex metabolic activation and detoxification by cytochrome P450 enzymes in the liver to liberate alkylating metabolites such as 4-hyroxycyclophosphamide/aldophosphamide, which serve as the transport species for the metabolites which can cross-link DNA (phosphoramide mustard and acrolein) (Chen et al., 1997). 4-hydroxyclophosphamide and aldophosphamide also undergo enzymatic oxidation detoxification to form inactive urinary excretion products 4-ketocyclophosphamide and carboxyphosphamide respectively (Chen et al., 1997).

Due to the toxic mode of action CP/IF could potentially induce genotoxic, teratogenic and mutagenic effects. Some suggest that the developmental vulnerability of an unborn child in the womb would be particularly susceptible to the teratogenic effects of chemotherapy drugs (Johnson et al., 2008). Major malformations, fetal death and spontaneous abortion have been reported with the use of CP during the first trimester (Cardonick and Iacobucci, 2004). Prognosis is better if CP is delivered during the 2nd and 3rd trimester where a review indicated that this exposure carries little risk of malformations (Amant et al., 2012)

Several authors have reviewed the considerable number of anticancer drugs used in chemotherapy, which when discharged may have harmful consequences for the environment (Booker et al., 2014, Johnson et al., 2013). Booker et al. (2014) highlights the highly consumed anticancer drugs in the North West (NW) England, which provides accurate consumption data and use patterns for the UK. Precise consumption data allows predictions on influent concentrations to be made to help corroborate analytical studies. Previous attempts have been made to predict the concentrations of anticancer drugs in the environment, where calculations were done from a theoretical point of view. The aims of this study was to comprehensively understand the occurrence and fate of CP and IF in STP influents, effluents and receiving waters; examining a range of STP plants that serve different population densities and possess different types of water treatment. This will allow an assessment of the removal efficiency of different STPs as well as providing valuable field data to determine chemical loads to surface waters. Furthermore, surface water concentrations of CP and IF are sampled in a defined river catchment in NW England (River Ribble). Measured river water concentrations together with previous calculated theoretical loads can be correlated as a tool for future predictions in catchment areas for anticancer drugs.

2. Types of sewage treatment

Sewage treatment in England is managed by several private companies each with a regional base. This study conducted sampling at STPs from the following four companies United Utilities Plc (NW England), Severn Trent Water Ltd (Midlands), South West Water Ltd and Thames Water Utilities Ltd (London and Greater London). Table S1 (Supplementary information) summarizes data from the Water Services Regulation Authority (Ofwat) 'June returns, Chapter 17 (2009)' for the investigated

sewage works. The Ofwat classification for STPs classifies all treatment plants into 7 discrete categories, suitable for discriminating between treatment processes in terms of pollutant removal efficiency.

3. Materials and Methods

3.1. Sampling

Wastewater samples were obtained from 14 STP located across North, South and East England over the period 2011-2012. The details of these STP types are described in Table S1 These plants operate 2 to 3 stages of wastewater treatment, involving physical separation of solids (primary treatment (P)) and secondary treatment (secondary activated sludge (SAS) or secondary biological (SB)). 71% of the STPs sampled had a third stage of wastewater treatment either Tertiary A2 (TA2) or Tertiary B2 (TB2) whose treatments include rapid-gravity sand filters, moving bed filters, pressure filters, nutrient control, disinfection hard COD and colour removal. TA2 works with secondary activated sludge plants and TB2 works with secondary biological process. Details of each STP installation, population equivalent and consents are provided in Table S1.

The timings of sample collection involved in this project were considered, grab samples were collected between 10am and 2pm on weekdays to catch the morning sewage and to account for the CP and IF that is administered during outpatients clinics. In the first sampling campaign (locations 7 to 14) influent and effluent wastewater samples were collected proportionately to the flow over 24 hours 'composite' samples (nano-filtered and frozen at -20°C on return to lab) and in the second sampling campaign (locations 1 to 6) influent and effluent samples were collected as 2.5L grab samples and stored at 4°C prior to extraction (extraction was

conducted within 48 hours of sample collection). STP locations and the corresponding river catchment located in NW UK are shown in Figure 1. Sample site 7 was sampled twice; both samples obtained were 24hr integrated samples. Sampling was conducted during low-normal flow conditions with the exception of sampling site 10, which was conducted during a period of higher flow (≥100mm of rainfall per month)(Table S3).

The river catchment study was carried out in NW England, centered on the River Ribble (the main river within the catchment) with a length of 194 km, draining a basin of 2227 km^2, home to 1.25 million inhabitants. In parts the three predominant rivers, the Ribble, Darwen and Calder are densely populated and flow through large urban towns such as Preston, Blackburn and Burnley, respectively, with the rest of the catchment (90%) being predominately rural. The STP influent and effluent samples from locations 1 to 6 are located within the River Ribble catchment, a further six samples were selected on the Rivers Calder, Darwen and Ribble, largely close to the effluent discharge point (from a minimum of 50 m to a maximum of 11.3 km from the STP effluent discharge point), with further details presented in Table 1. Details of the six STP plants are listed in Table S1 and their locations are shown in Figure 1. Sample site 5 was visited twice, where one 2.5 L grab sample and one 24 hr time integrated sample was obtained. The river sample corresponding to site 6 is a sample from the River Ribble in Preston (11.3 km downstream from the effluent discharge at site 8 and 40 km from the effluent discharge at site 3), detailed in Table 1. The effluent water from site 6 (downstream of river sample) is discharged into a tidal estuary, draining into the Irish Sea.

3.2. Sample preparation and chemical analysis

Chemical standards of cyclophosphamide monohydrate (2-[Bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide) and ifosfamide (N,3-Bis(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine-2-oxide) were obtained from Sigma Aldrich (Gillingham, Dorset, UK) with chemical structures provided in Table S4 (Supplementary information). Custom-synthesized internal standard (IS) of deuterated-cyclophosphamide (d4-cyclophosphamide or d4-CP) was purchased from Qventas Laboratories (Branford, CT, USA), with HPLC-grade solvents purchased from Rathburn chemicals (Walkerburn, Scotland).

Preparation and treatment of both time integrated and grab samples followed a previously described method (Llewellyn et al., 2011). In brief, 500 mL of each sample was initially filtered (GFF filters, Whatman, UK) and spiked with 5 μL of d4-CP IS for quantification purposes (resultant concentration approximately 15 ng/L). The filtrate was passed through a Strata-X (Phenomenex, Macclesfield; UK) SPE cartridge and eluted with 10 mL of HPLC-grade ethyl acetate. The ethyl acetate extract was then eluted through 200 mg of Florisil (Biotage, Uppsala; Sweden) and the resulting eluent concentrated under N_2 and made upto 500 μL with the LC mobile phase (comprising ~95% water, ~5% methanol ~0.1% formic acid) and quantitatively transferred to 2 mL autosampler vials for analysis. All standards and extracts were stored in amber vials at -20°C prior to instrumental analysis.

Analyses were performed on a LC-MS/MS instrument: an Accela high performance liquid chromatograph (Thermo Fisher Scientific Inc) coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific, Hemel Hemsptead; UK) interfaced with an ion max electrospray ionisation (ESI) and

operated with Xcalibur software ™ (V.2.0.7.) Analyte separation (10 μL inj. volume) was performed on a μPLC Hypersil GOLD C18 column (50 x 2.1 mm 1.9 μm) using a H2O:MeOH mobile phase gradient (Llewellyn et al., 2011). Seven calibration standards ([IF] = 0.14-6.63 μg/L, [CP] = 0.22-10.33 μg/L, [d4-CP] = 5 μg/L) were freshly prepared in the mobile phase solvents for each analysis (Table S5 shows the prepared concentrations for CP and IF calibration) with the addition of 5 μL IS d4-CP to each sample and calibrant (resultant concentration 15 ng/L). Instrumental operating conditions are provided and in Table S6, with IF eluting at 5.34 minutes and CP at 5.74 minutes with baseline separation for the two compounds. Chromatographic peaks were integrated using the ICIS algorithm of Xcalibur™ which was also used to generate linear calibration curves using a 1/X weighting. Analyte peak signal to noise (S/N-RMS) ratios were obtained with the manual noise region option in Xcalibur™ .

3.3. Quality control and assurance

CP and IF calibration standards were freshly prepared for each analysis with calibration standards bracketed around no more than eight unknown samples and as such injected at least twice during each analytical run. All samples and standards were spiked with IS d4-CP (Llewellyn et al., 2011). The LC-MS/MS instrument was initially run with pure MeOH prior to each analytical run with mobile phase blanks (both with and without d4-CP) used to check for analyte carry over and purity-check of the IS. Calculations were performed using area ratios of CP and IF on the IS (d4-CP). Ionisation of CP and IF was conducted via heated ESI (HESI), however, samples collected from locations 1-6 suffered from lower recoveries of the IS compared to samples collected from locations 7 to 14. Therefore atmospheric pressure chemical ionisation (APCI) was also tested, under both positive and negative polarity with

comparison of the two ionization modes (HESI and APCI). Likewise calculations were performed using area ratios of CP and IF on the IS (d4-CP).

3.4. Recovery

Recovery of IS d4-CP was ~20% for APCI (+) and > HESI (+) recovery. In general the average recovery for sample sites 1 to 6 using HESI was 41.0% for STP influent samples and 30.5% for effluent samples, whereas, the average recovery for the same samples using APCI was 82.3% (influent) and 37.3% (effluent). The lower HESI and effluent wastewater recoveries were perhaps due to matrix effects associated with the nature of the wastewater samples. Signal suppression in ESI was significantly more pronounced than for APCI for CP, IF and d4-CP. It has been proposed that ion suppression mainly involves changes in the droplet solution properties caused by the presence of non-volatile solutes in ESI of complex extracts, rather than gas phase reactions leading to the loss of net charge on the analyte that may occur in APCI (King et al., 2000). APCI provided a better overall recovery but with slightly higher instrumental limits of detection (LOD). MDLs for CP ranged between 0.03 - 0.12 ng/L, and between 0.05 - 0.09 ng/L for IF. The detected concentrations were far greater than the LOD and despite the increase in IS recoveries for samples run using APCI, the concentrations calculated for both ionisation modes were comparable.

4. Results and Discussion

The first approach in 1996 measures CP in hospital effluent using gas chromatography-mass spectrometry (GC-MS) with CP derivatives for internal standardization described in Steger-Hartmann et al. Previous attempts to quantify CP with GC-MS have reported limits of detection (LOD) of 6ng/L for CP. However, it is now the common approach to quantify with liquid chromatography-tandem mass

spectrometry (LC-MS/MS) using a triple quadrupole (Q1q2Q3). These methods give detection limits below 2ng/L (Castiglioni et al., 2005, Yin et al., 2010), with two methods reporting detection limits in the sub ng/L range (Llewellyn et al., 2011, Buerge et al., 2006). A review of the methodology for sampling, extracting and quantifying CP in the aquatic environment is detailed in Table S2 (Supplementary information).

4.1. Cyclophosphamide/Ifosfamide in STPs

CP was detected in 93% of wastewater influent and effluent samples, frequency of detection can be seen in Table 2. For grab sampling, three discrete samples were collected at each STP site and results combined to provide an average concentration. However, IF was only detected in one wastewater influent site (no. 12) and two wastewater effluent sites (no. 6 and 7). This low frequency of detection is shown in Table 2 and is likely to be attributed to the low UK consumption of IF. The lower frequency of detects and lower concentrations of IF relative to CP could be attributable to consumption patterns, only 50% of the NHS trusts surveyed in NW England consumed IF compared to 100% for CP (Booker et al., 2014).

The CP concentrations were the highest with the influent and effluent concentrations ranging 0.43 -18.02 and 0.09 – 22.69 ng/L, respectively, whilst IF wasn't detected (<0.12 ng/L) for influent wastewaters and <0.12 – 0.77 ng/L for wastewater effluents (Table 2 and S3). The STPs with the highest concentrations serve the major conurbations of Preston (population ~ 247,000), Fazakerly (population ~ 175,000) and Blackburn (population ~ 289,000) (Site numbers 6, 7 and 5 are shown in Figure 1). The concentrations for both CP and IF are several orders of magnitude lower than

those at which acute ecotoxicological effects have been reported to occur (mg/L) (Kümmerer et al., 1997).

The CP concentration in STPs worldwide vary widely from <LOD to 13,100 ng/L in influent wastewaters, with the highest concentration reported in Spain, 2012 (Gómez-Canela et al., 2012). For effluents CP ranges from <LOD to 20 ng/L, with the highest concentration reported in Germany, 1998 (Ternes, 1998). Whereas IF concentrations range within <LOD to 29 ng/L in wastewater influent from a variety of studies (Buerge et al., 2006), with the highest concentration being reported in Germany, 1997 (Steger-Hartmann et al., 1997) and range from <LOD to 2,900 ng/L in wastewater effluents, with the highest concentration reported in Germany, 1998 (Ternes, 1998). The per capita consumption of CP and IF in England in 2011, was approximately 40 and 1 μg/cap/d, respectively. The European mean per capita consumption of CP between 1997 and 2011 was ~10 μg/cap/d (Booker et al., 2014, Johnson et al., 2013). The per capita consumption of CP and IF in Spain (2010) was approximately 75 and 20 μg/cap/d (~2 – 20 times > consumption in the UK) explaining the wide variation in measured CP and IF in STP plants internationally. Several studies have measured concentrations of CP in STP effluents, with concentrations ranging from 2.1 – 4 ng/L in Zurich (2006), 12 ng/L in Montreal STP (2011), 2.5-4 ng/L in another Canadian study in 2006 and concentrations < 1 ng/L in Italian and Spanish studies (Buerge et al., 2006, Gómez-Canela et al., 2012). A UK study reported CP between 0.2-3.6 ng/L in two STP plants (Llewellyn et al., 2011). The measured values in this study are within the predicted and detected ranges for CP (Booker et al., 2014) (Mean influent/ effluent CP concentration are 4.1 and 6.6 ng/L, respectively).

Booker et al 2014 predicted CP to be present in raw influent waters to be 41.5 ng/L. This is 10 fold higher than the average UK measured CP value in this study, this value falls outside of the measured range perhaps due to; (1) an assumed consumption rate derived from hospitals with higher than average CP concentration that is not comparable to the other areas sampled in the UK, (2) chemotherapy is often prearranged during outpatient clinics and so CP and IF might show daily concentration variations in STPs, (3) the modelled value was derived from predicted biodegradation rates and a set dilution per head of 200 L/day, where in fact the dilution to STPs may vary and be greater than this set value. STPs that receive hospital wastewater (large oncology unit) or densely population areas display concentrations closer to the predicted concentration (sites 5, 10 and 12). For example, the highest influent concentration detected in this study was 18.02 ng/L; the sample was measured from a sewage works that received the largest daily load of all the surveyed sites (site number 5).

4.2. Cyclophosphamide behaviour during wastewater processing

The concentrations detected in the effluent wastewaters were on average > 2-fold higher than raw influent water. The overall removal rate was calculated for all the composite time integrated samples during wastewater treatment (grab samples were omitted to evade discrepancies associated with any diurnal variation of CP levels). CP investigated in this study exhibited very different removal efficiencies, ranging from -433% to 83% and the efficiency of the removal varied from one treatment plant to another. Figure 2 shows a summary of concentrations comparing influent and effluent wastewaters. There was a significant difference in the concentrations for the influent (mean 3.78±3.25 ng/L) and effluent (mean 8.72±6.94 ng/L) for the plants which operated a tertiary treatment process; $t(9) = -2.27$, $p = 0.05$ (Fig 2c) but not for the

other types (Fig 2a and 2b). These results demonstrate that the effluent waters contain higher concentrations of CP than the corresponding influent, particularly for plants which operated a tertiary treatment.

The increase in the concentration of CP, namely "negative removal" during treatment, is a phenomenon reported in other studies for some pharmaceuticals and personal care products (PPCPs) (Besse et al., 2012, Ortiz de García et al., 2013, Yan et al., 2014). The chemical structure seems to be an important factor in determining removal rates. Ternes et al., 1998, demonstrated that medium polarity drugs are removed on average between 60% - 90%, whilst polar PPCPs exhibit lower removal during wastewater treatment (lower adsorption onto activated sludge particles). Diclofenac is an example of a PPCP (log K_{ow} = 4.51) that has been recovered in effluent wastewaters upto 95%, with some studies showing higher concentrations in the effluent because of desorption processes (Quintana and Reemtsma, 2004). CP is more polar than diclofenac and has a log K_{ow} of 0.63 (Booker et al., 2014).

This phenomenon of higher effluent concentrations may be attributed to the regeneration of CP during treatment could be related to deconjugation, a phenomenon frequently described for estrogens (Kumar et al., 2012). Whereby the glucuronide conjugates allow the complete transformation to the free parent compound within a sewer, preferably favourable in the STPs operating tertiary treatment with longer retention times. Li et al., 2010 demonstrate a minor CP glucuronide (alcophosphamide glucuronide) conjugate detected from CP treated mice, it is plausible that this type of compound could regenerate parent CP during sewage treatment.

4.3. Release of cyclophosphamide in receiving waters and chemical loads

Table 2 presents the concentrations of CP measured in the rivers Calder, Darwen & Ribble located in NW England. The concentrations of CP detected in the STP influent and effluent wastewaters were multiplied by the average daily flow rate at each sampling site to obtain environmental loads, expressed in mg of CP per day (Table 1).The sites are illustrated in Figure 1b and distances downstream from STPs are recorded in Table 1. CP was present at 5 of the 6 sampling sites, and IF was not present above the MDLs.

With limited data available on the concentrations of CP and IF in UK waters, a modelling approach is desirable in order to quantify loads to river water from STP discharges and to verify existing modelling approaches. Booker et al., (2014) details the hospital consumption data for CP and IF for the hospitals located within the River Ribble catchment, whereby 18.1 kg/yr of CP is consumed (assuming a catchment population of 1.25million). Approximately a 21-fold dilution occurs within the catchment from effluent discharge point to the river water under low flow/normal conditions. Assuming an average human excretion rate for CP of 21% (Booker et al., 2014) and considering that the STPs captured waste from 68.5% of the catchment population (1.25million), 1.8 kg/year is consumed within the Ribble catchment, of which 4.5% was present in the STP influents, 3.2% in the effluents and 2.4% in the river water (at site 6; after the confluence of the Calder and Darwen). We predicted CP concentrations would be within the ranges of 0.01 – 0.27 ng/L for this study, calculated from the measured effluent loads.

CP was detected in river water samples between <LOD to 3.78 ng/L, with a mean of 1.36 ng/L (±1.49 as shown in Table 3). Both values are consistent with previously

published data that predicted river water concentrations in North West England downstream of STPs to be 4.1 ng/L (Booker et al., 2014), and nationwide in France at >1.75 ng/L in sewage effluent which would equate to 0.18 ng/L in surface waters using a 10-fold dilution from effluent to river water (Besse et al., 2012).

Figure 3a illustrates the detected loads of CP for the River Calder (Sites 1, 2 & 3) (also illustrating the corresponding STP influent and effluent values). Figure 3b illustrates the detected loads of CP for the River Darwen (Sites 4 & 5) (also illustrating the corresponding STP influent and effluent values). Figure 3b shows each river with an increasing CP load down the catchment, both rivers flow into the River Ribble and the final site is located after the confluence (Site 6). The sites show an increase of CP from influent to effluent in all sites except site 5 (located on the River Darwen). This increase load is due to a high measured influent value principally due to sampling protocol and diurnal variation of CP. The final river water sample at site 6 is located 40 km from the previous STP on the River Calder and 11 km from the previous STP on the River Darwen. River site 6 has the greatest load detected in the Ribble catchment, with a measured value of 0.41 ng/L (±0.08) and large river flow of approx. 2.89E+09 L/day (largest river flow in catchment), thus producing a high environmental load and illustrating that CP is maintaining within the catchment.

To our best knowledge these data are the first which evaluate cyclophosphamide in a river catchment study, showing that cyclophosphamide does reach surface waters and shows accumulation in the rivers downstream of STPs. This may cause implications at times of low flow, where the subtle effects associated with low dose exposure will be an issue that requires further attention, particularly for anticancer drugs with high cytotoxic potency (Booker et al., 2014, Strong, 2012). In order to assess the

magnitude of the problem which is caused by release of anticancer drugs in environmental compartments, further toxicity testing at river level concentrations needs to be carried out with a larger array of chemicals.

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Table 1: Environmental loads expressed in mg of CP per day. Sites 1 to 6 are contained within the River Ribble catchment (NW England).

Location	STP flow L/day	River flow L/day*	STP Population equivalent	CP influent load (mg/d)	CP effluent load (mg/d)	CP river load mg/d (distance downstream)
1	5.80E+06		2.90E+04	2.67	1.97	<LOD (200 m)
2	2.70E+07	2.53E+08[1]	1.35E+05	11.64	32.24	58.22 (1.2 km)
3	2.44E+07	7.46E+08[2]	1.22E+05	12.59	122.71	432.42 (2.0 km)
4	6.80E+06	6.80E+06[3]	3.40E+04	11.90	93.18	25.70 (50 m)
5	5.78E+07	3.67E+08[4]	2.89E+05	1041.79	313.62	659.87 (1.3 km)
6	4.94E+07	2.89E+09[5]	2.47E+05	251.99	374.80	1183.76 (11 km from STP 8 and 40 km from STP 3)
7	3.50E+07	-	1.75E+05	135.90-317.37	55.35 – 253.17	
8	8.20E+06	n/a	4.10E+04	30.70	33.14	
9	4.20E+07	n/a	2.10E+05	22.09	46.34	
10	3.98E+07	n/a	1.99E+05	367.91	653.63	
11						
12						
13	5.80E+06	n/a	2.90E+04	5.30	18.85	
14	1.10E+07	n/a	5.50E+04	<LOD	1.00	

*Data from the National River Flow Archive, CEH, river flow for day of sampling (or thereabouts)

[1] 71010 – Pendle water at Barden Lane

[2] 71004 – Calder at Whalley Weir

[3] Gauge station (71013) data not used, at point of sampling river flow was equal to STP flow

[4] 71014 – Darwen at Blue Bridge gauge station

[5] 71001 – Ribble at Preston gauge station

Table 2: Summary of all measured CP and IF concentrations, including the frequency of detections for sampled influent and effluent wastewaters.

	Cyclophosphamide (ng/L)		Ifosfamide (ng/L)	
	Influent	Effluent	Influent	Effluent
Median	1.75	3.65	NA	0.33
Mean	4.18	4.83	NA	0.38
Min	<LOD	<LOD	<LOD	<LOD
Max	18.02	16.42	<LOD	0.77
SD	5.21	5.03	NA	0.29
Frequency (%)	93%	100%	0%	29%

Values identified as < LOD are below the detection limit and not detected.

Table 3: Cyclophosphamide concentrations (ng/L) measured in triplicates in different influent, effluent and river water in the River Ribble catchment (NW England).

	Cyclophosphamide (ng/L)		
Sampling Location (Figure 1b)	Influent	Effluent	River
1	0.46±0.03	0.344	<LOD
2	0.43±0.03	1.19±0.32	0.23±0.18
3	0.52±0.09	5.03±1.70	0.58±0.12
4	1.75±0.06	13.70±1.41	3.78±0.21
5	18.02±1.87	5.43±0.14	1.80±0.16
6	5.10±0.22	7.59±0.54	0.41±0.08
Median	1.13	5.23	0.58
Mean	4.38	5.55	1.36
Min	0.43	0.34	<LOD
Max	18.02	13.70	3.78
SD	6.92	4.84	1.49
Frequency (%)	100%	100%	83%

Values identified as < LOD are below the detection limit and not detected.

Figure 1: (a) The sampled STPs in England and the daily volume of sewage treated plotted on a population density map from census survey 2011. (b) River Ribble catchment sampling locations.

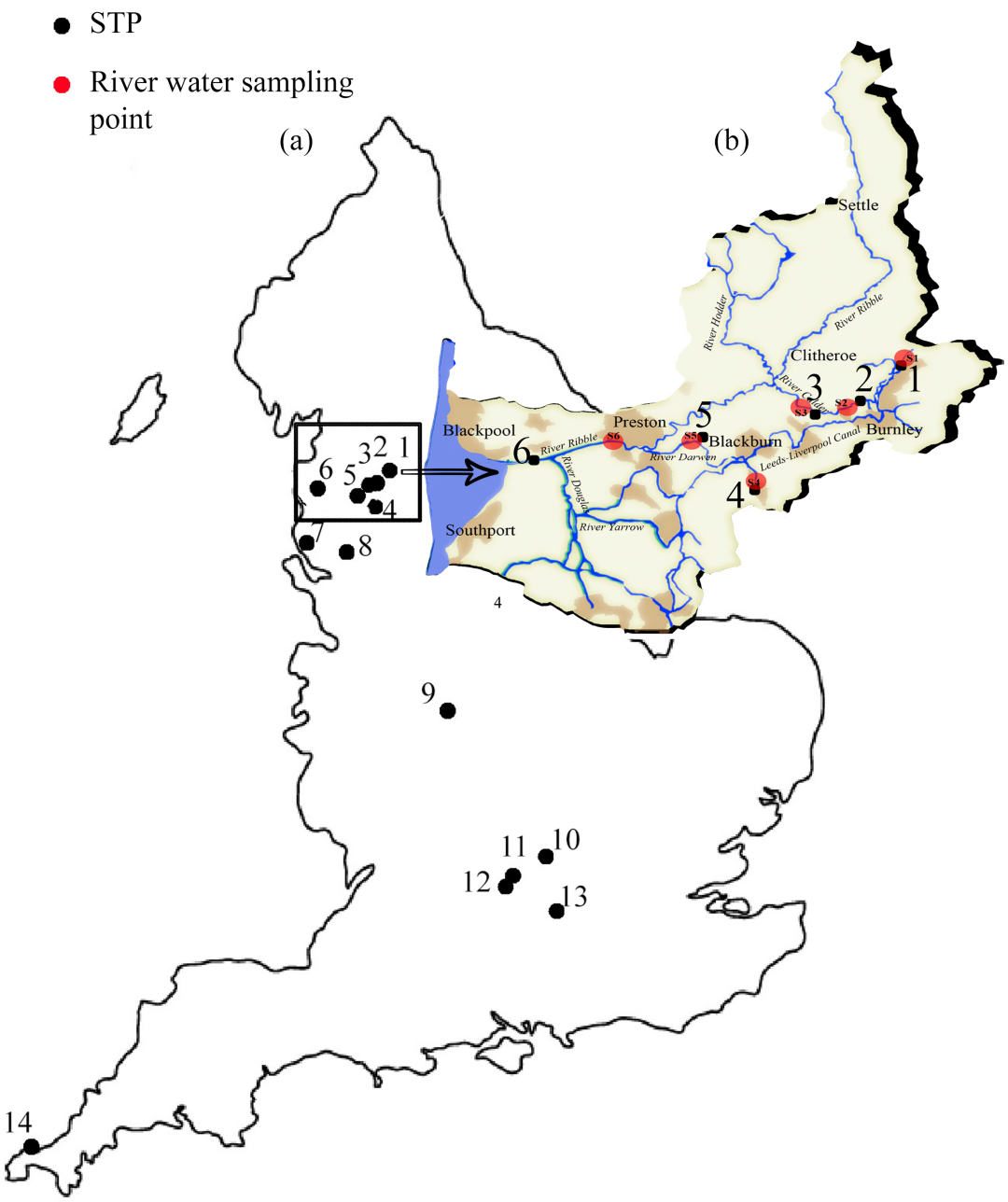


Figure 2: Summary showing the strength of the difference in CP concentration in the influent vs. effluent wastewaters for different samples. (a) Includes data from all STPs (n=16) in the study. (b) Includes data from STPs (n= 10) that utilise both secondary and tertiary treatments.

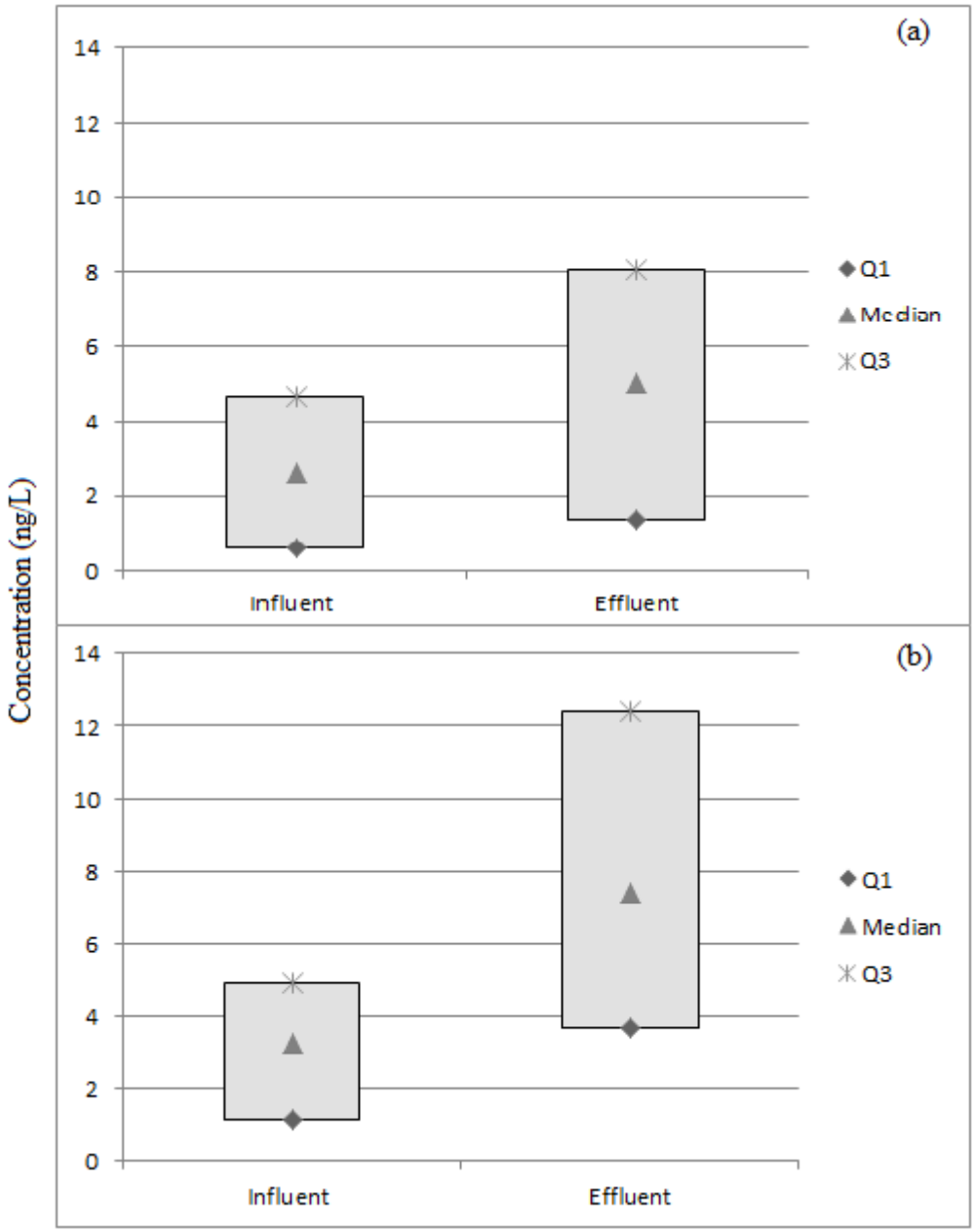
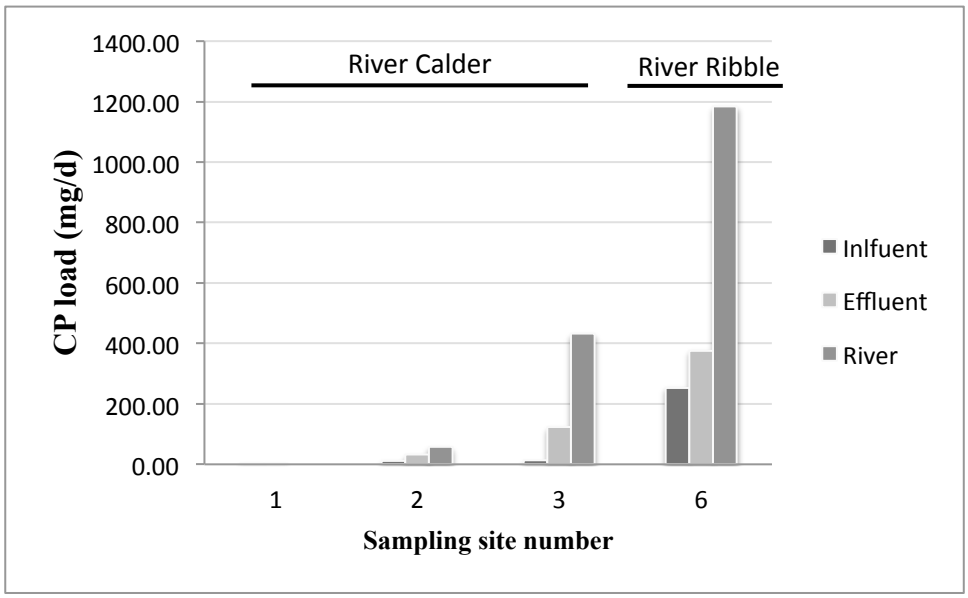
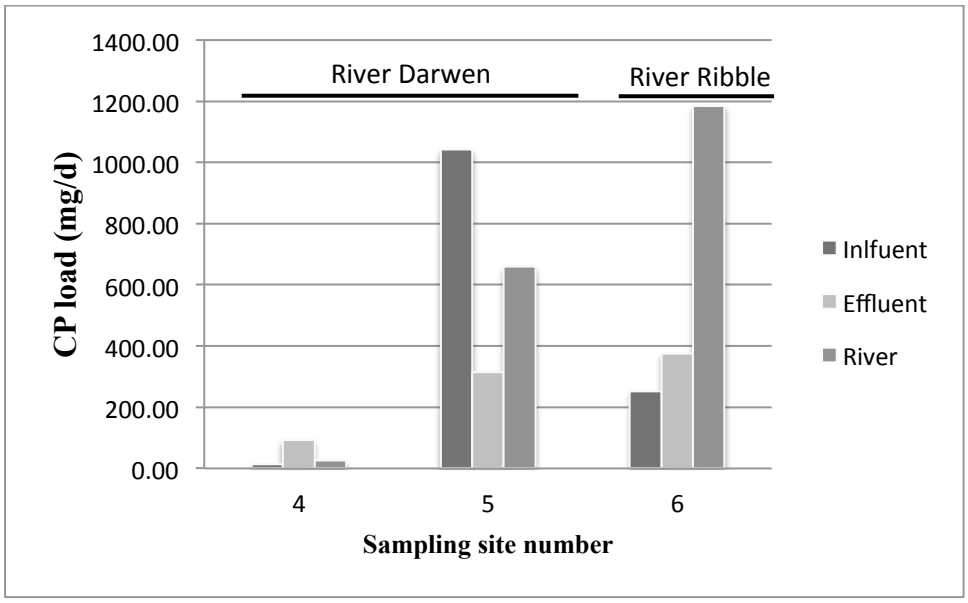


Figure 3: Environmental loads (mg per day) of CP within the Ribble catchment for influent, effluent and river water samples where (a) shows the River Calder flowing into the River Ribble and (b) shows the River Darwen flowing into the River Ribble.



(a)



(b)

Table S4: Chemical information on drugs analyzed and their applications

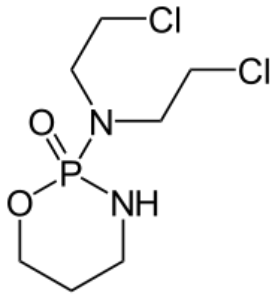
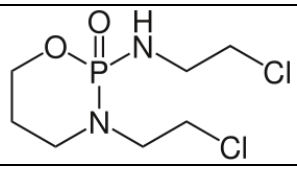
Compound	Structure	Molecular Weight	pKa	Log K_{ow}	Application
Cyclophosphamide		261	2.84, 6.00	0.63	Nitrogen mustard alkylating agent; attaches to the guanine base of DNA. Used to treat lymphomas, some forms of brain cancer, leukaemia and some solid tumours.
Ifosfamide		261	1.45-4.00	0.86	Nitrogen mustard alkylating agent; used in a variety of cancers.

Table S5: Calibration ranges

	CP ng/L (pg/µL)	IF ng/L (pg/µL)
L1	0.22	0.14
L2	0.43	0.28
L3	0.86	0.55
L4	1.72	1.11
L5	3.44	2.21
L6	5.16	3.32
L7	10.33	6.63

Table S6: MS-MS Ion source parameters

Parameter	HESI
Polarity	Positive
Spray voltage (V)	3000
Vaporiser temperature (°C)	350
Sheath gas pressure (arbitrary units)	30
Ion sweep gas pressure (arbitrary units)	0
Auxiliary gas pressure (arbitrary units)	30
Ion transfer capillary temperature (°C)	300
Collision gas pressure (mTorr)	1
Skimmer offset voltage (V)	-5

Supplementary Information References

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Paper III

Towards a mass balance of the common anticancer drug, cyclophosphamide, during wastewater treatment and release to receiving waters

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KEYWORDS

Pharmaceuticals; anticancer drugs; sewage; influent/effluent; elimination; cyclophosphamide

Abstract

The fate and removal efficiency of cyclophosphamide (CP) and ifosfamide (IF) were investigated in two conventional sewage treatment plants (STP-S and STP-C) in North West (NW) England during different stages of wastewater treatment. Overall average concentrations of CP were 1.17±1.00 ng/L (n=32) in the two plants, which is lower than recent measurements conducted elsewhere. Whereas IF was below MDLs in both plants. Grab-samples were coordinated with the hydraulic residence time of wastewater in each of the treatment stages in order to monitor changes in CP concentrations in the same parcel of water as it passed through the STP. Interestingly, concentrations of CP were observed to increase from raw influent to final tertiary-treated effluent and this is likely to be attributable to the degradation of a CP-metabolite and subsequent 'liberation' of the parent CP as the metabolite passes through the various sewage treatment processes. This observation, apparent in both studied STPs, has implications for chemical fate modelling of anti-cancer drugs, especially if STP influent loads are used to predict subsequent fluxes to receiving waters rather than final effluent values. Moreover, this increase in concentrations made a mass balance difficult to achieve, but highlighted that elimination/removal of CP in wastewater during primary to tertiary processing is very low (<20%). The calculated fluxes of CP with final effluent discharge were 3.16- 6.48 g/year for STP-S and 4.56 -51.57 g/year for STP-C and this highlights that STPs are a continuing source of highly water-soluble, recalcitrant anticancer drugs to the environment.

1. Introduction

Pharmaceuticals chlorophenoxyisobutyrate and salicylic acid (metabolites of clofibrate and aspirin) were first detected in the aquatic environment by gas chromatography-mass spectrometry (GC-MS) in the effluent wastewaters of a sewage treatment plant (STP) in Kansas City, Missouri (1977) (Hignite and Azarnoff, 1977). Since then detection of 'down the drain' pharmaceuticals that reach the environment and have environmental significance have been increasingly reported in STP effluents (Andreozzi et al., 2003, Nefau et al., 2013, Kumar et al., 2012).

Anticancer drugs are used in medical oncology as a method of treating a range of cancers, where one or more agents are given as part of a standardized chemotherapy regime. An aging population has seen an increase in their consumption, where they are administered during chemotherapy with a curative intent, or in an aim to prolong life and reduce patients' symptoms (Johnson et al., 2008, Booker et al., 2014, Besse et al., 2012). Cyclophosphamide (CP) and Ifosfamide (IF) are widely used traditional anticancer agents in the UK, as well as other European countries and have aroused concern within the scientific and regulatory community with respect to their environmental release and potential hazards due to their low dose cytotoxic effects (Booker et al., 2014). Particular concern has arisen due to the persistence of some of these chemicals and the risk they pose to drinking water, particularly where river water is abstracted downstream of STPs for water treatment and a potable water supply (Johnson et al., 2008). Chronic ecotoxicity is infrequently reported for anticancer drugs, but where literature is found the concentrations of chronic toxicity occurs at more environmentally relevant concentrations (ng/L rather than mg/L) (Parrella et al., 2014). Some anticancer agents have been found to exhibit low toxicity in standard eco-toxicity tests with aquatic organisms (e.g. 5-fluorouracil has an EC_{50}

value of 0.11 mg/L for a species of algae *(Pseudokirchneriella subcapitata)* (Zounkova et al., 2010) and methotrexate has an EC_{50} of 0.015 mg/L for the African clawed frog *(Xenopus laevis)* with similar values reported for fish (*B. Rerio)* (Bantle et al., 1996, Henschel et al., 1997)), but in real aquatic environments that receive wastewater effluents anticancer drugs will appear as mixtures alongside metabolites, transformation products and other pharmaceuticals and it's possible that some of these chemicals will act synergistically with effects (Parrella et al., 2014).

The fate of anticancer drugs during wastewater treatment processes has not been studied and yet is likely to provide insight into how some of these chemicals can carry over to final effluent, be degraded during secondary treatment processes or lost with sewage sludge. Sewage treatment plants (STPs) are designed to reduce the load of organic material and pathogens present in raw wastewater; however, they are not designed to specifically remove pharmaceuticals or other organic pollutants (Joss et al., 2005). Most STPs involve physical-chemical mechanisms of treatment (e.g. flocculation and activated carbon adsorption); however this only provides marginal removal of some pharmaceuticals (Suarez et al., 2009) with biological methods known to provide the most effective elimination for pharmaceuticals. For example, aerobic activated sludge (AS) with long hydraulic retention time (HRT) is a frequent treatment option (Oz et al., 2004) and is the most widely used in sewage treatment processes across Europe. However, sorption onto the bio-sludge and biodegradation under aerobic conditions results in < 2% removal for CP and IF (Booker et al., 2014). Therefore, CP and IF degrade very slowly, with little if any degradation occurring during the whole sewage treatment process. Previous literature shows that little is known about the fate of these chemicals in STPs, with most studies relying on estimates from modelling programmes, such as EPISUITE (Dong et al., 2013).

Indeed, very low (bio)degradation of CP and IF was demonstrated in effluent samples incubated in the dark for 28 days, resulting in experimental half lives of 495 and 577 days, respectively (Llewellyn et al., 2011). The long half-lives and low degradation rates highlight that CP and IF are persistent in STPs and receiving waters.

The occurrence of CP and IF in STPs has been reported in a number of studies in different countries (Buerge et al., 2006, Garcia-Ac et al., 2009, Castiglioni et al., 2005, Gómez-Canela et al., 2012, Martín et al., 2011), however these studies show a lack of information about the removal efficiency and release of CP and IF in STP effluents. Few (if any) studies have considered the fate and distribution of anticancer drugs throughout each separate stage of the sewage treatment process in order to comprehensively evaluate the biodegradation, persistence and partitioning behaviour in both aqueous and solid phases (Llewellyn et al., 2011). Using flow rates and measured concentrations at each phase of the sewage treatment process the aim of this study was to determine the fate and fluxes of CP and IF from raw influent to final treated effluent in two different STPs. More specifically, the major objectives of this study were to (1) evaluate the removal efficiencies of CP and IF in two major STPs, (2) compare the capabilities of the two STPs in removing CP and IF, at each stage of treatment and (3) characterize the profile of CP and IF by quantifying its percentage removal throughout sewage treatment.

2. Materials and methods

2.1. Site description and treatment plant operation

Two STPs located in NW England were selected for this study; one located near the city of Lancaster (STP-S) of 32 - 72.5 ML/day (1 ML = 10^3 m^3) capacity (maximum flow to full treatment capacity = 87 ML/day) and the other near the city of Preston

(STP-C) receiving 104 ML/day capacity (maximum flow to full treatment capacity = 234ML/day). Arial photographs of both sites and their systems in place are shown in Figure 1 and their main operational and design parameters of the full scale STP are summarized in Tables 1 and 2. In both STPs, sewage from smaller towns reach the STPs after multistage pumping because of the distance and flat topography of these cities; wastewater enters the works through pumping stations whereas at the treatment sites wastewater flow is maintained by gravity. Following gravitational primary treatment, the settled sewage effluent is introduced into aeration lanes (STP-S) or tanks (STP-C) (activated sludge) for secondary treatment. After settling the effluent undergoes a tertiary treatment phase (final settling tanks), before the effluent is subjected to UV-light and released into tidal storage tanks (STP-S) or into the nearby recipient river estuary (STP-C).

Plant 1; STP-S serves a population equivalent of 27,698 and receives hospital waste from the oncology unit at Royal Lancaster Infirmary (population served =363,000). Royal Lancaster Infirmary provides CP and IF as part of their chemotherapy regimens and is consumed within this hospital trust at 1300 g/year and 150 g/year, respectively. Plant 2; STP-C serves a population equivalent of 247,000 and receives hospital waste from the oncology unit located within Royal Preston Hospital (population served = 390,000). Royal Preston Hospital (Rosemere cancer foundation) provides only CP as part of its chemotherapy regimens and is consumed at approximately 610 g/year. A neighbouring hospital that serves a population of 330,000 may also contribute to the CP load in STP-C as patients attend outpatient clinics and disperse home after treatment. Only CP is consumed within this hospital at approximately 1900 g/year. The hospitals within the catchments areas for both the STPs investigated rarely use IF

as part of their chemotherapy regimen and therefore its presence in the environment is expected to be negligible for this specific region of the UK.

2.2. Sample collection

The sampling programme was designed so that wastewater samples were collected to reflect the HRT within each of the STPs. Samples of raw influent and subsequent samples following primary, secondary, tertiary treatment and final effluent, therefore reflected the same parcel of water, where possible, as it progressed through the treatment plant. Table 2 presents the flow characteristics and HRT for each of the major components of the respective plants.

Samples in STP-S were collected in October 2013, with grab sampling conducted on 8th October 2013 and 24-hr composite samples collected over a period of seven days from 22nd to 28th October 2013. 24-hr composite samples were collected from three sampling points, using fixed sampling units; (a) 'raw' influent (i.e. crude sewage) (b) Mid-treatment (Primary effluent) and (c) Pre-UV treatment. STP-S details are presented in Table 1. Grab samples were collected from six sampling points: (a) Influent was taken as a mixture of multiple grab samples after preliminary treatment (i.e. screening and grit removal); (b) Primary effluent; (c) Aeration effluent; (d) Pre-UV treatment; (e) Post-UV treatment and (f) Storm return. The daily flow rate at the time of grab sampling was 72.5 ML/day and is representative of high flow in this plant, with a treatment HRT of 7 hours from raw influent to final effluent. Grab sampling details are presented in Table 2.

Samples in STP-C were collected in November 2014; grab sampling was conducted on 18th to 19th November 2014 with the first influent sample collected at 10am. Grab samples were collected from five sampling points: (a) influent (crude sewage) was

taken as a mixture of multiple grabs after the preliminary treatment (screening and grit removal); (b) Primary effluent; (c) Aeration effluent; (d) Pre-UV treatment and (e) Post-UV treatment. The daily flow rate during the sampling was 104 ML/day and is representative of low flow (dry weather) in this plant, with a treatment retention time of 25 hours from raw influent to final effluent. Due to the retention on the day of sampling, the samples obtained from the aeration effluent were representative of influent entering the plant at 4pm on 18th November 2014, and therefore the concentrations detected in these samples were disregarded in this study. Grab sampling details are presented in Table 2.

All wastewater samples were collected in 2.5L methanol rinsed amber glass bottles. All samples were transported immediately to the laboratory and stored in a dark room at 4°C as described by (Llewellyn et al., 2011).

2.3. Analytical methods

CP and IF were measured in samples of sewage wastewaters as described in a previously published method (Llewellyn et al., 2011). CP and IF in wastewater samples were extracted by a two-step solid phase extraction (SPE) technique. This involved a pre-concentration step using 500mg Strata-X SPE cartridges (Phenomenex, Macclesfield; UK) followed by elution through 200mg of Florisil (Biotage, Uppsala; Sweden) using ethyl acetate and ethyl acetate with 10% methanol, respectively. The resulting eluent concentrated under N_2 and made upto 500 µL with the LC mobile phase (comprising ~95% water, ~5% methanol ~0.1% formic acid) and quantitatively transferred to 2 mL autosampler vials for analysis. Analyte separation (10µL inj. volume) was performed on a packed µPLC Hypersil GOLD C_{18} column (50 x 2.1 mm 1.9 µm) using a H_2O:MeOH mobile phase gradient. Analysis was

performed by liquid chromatography coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead; UK) interfaced with an ion max electrospray ionisation (ESI) and operated with Xcalibur software ™ (V.2.0.7.). In this study all CP and IF concentrations are reported in ng/L.

Chromatograms were integrated using ICIS algorithm of Xcalibur™ 2.0.7 by Thermo Fisher Scientific and a linear analyte calibration curve was generated using 1/X weighing for six calibration standards each with a constant (5 µg/L) concentration of the internal standard (d4-CP). For each analysis sequence the calibration standards were bracketed around upto eight unknown samples with mobile phase blanks performed within the bracket to check for the carryover and the purity of the internal standard (IS). The system was initially cleaned with pure MeOH prior to each analytical run and calculations were performed using area ratios for both CP and IF to give recovery values of the IS. MDLs for CP ranged between 0.03 - 0.12 ng/L, and between 0.05 - 0.09 ng/L for IF.

2.4. Mass balance calculations

The average mass flux (F (g/year)) of CP and IF was calculated by multiplying the average aqueous concentrations (C_S (ng/L)) with the corresponding average daily flow (Q_S (L/day)). The equation can be expressed as:

Eqn. 1 $$F = Q_S \, x \, C_s$$

3. Results and discussion

3.1. Concentrations of CP and IF

A summary of measured CP concentrations is provided in Table 3.

Cyclophosphamide was detected and quantified in the majority of samples, whereas

IF remained below MDLs at both STPs. For CP, concentrations fell below MDL for raw influent grab samples collected during dry weather flow at STP-C, with the highest concentrations being observed in raw influent 24-hr composite samples at STP-S during a period of low flow. Daily 24-hr composite samples were obtained from STP-S over the course of a week and showed only moderate day-to-day variation in their concentrations of CP, IF was not detectable above the MDL (0.09 ng/L). CP was detectable in all 21 samples above the MDL (0.12 ng/L). The influent samples showed concentrations ranging between 0.14 to 4.31±0.04 ng/L (mean concentration = 1.56±1.31 ng/L). The primary effluent samples showed concentrations ranging between 0.30±0.06 to 3.52±0.21 ng/L (mean concentration =1.61±1.08 ng/L). The final effluent wastewaters after UV treatment showed concentrations ranging between 0.88±0.08 – 2.09±0.05 ng/L (mean concentration = 1.49±0.64 ng/L). Results are illustrated in Figure 2 and Table 3.

It is well established in the literature that STPs are the most important contributor of anticancer drugs to the environment (Buerge et al., 2006, Thomas et al., 2007, Martín et al., 2011, Llewellyn et al., 2011, Kümmerer et al., 1997). The two studied STPs (STP-S and STP-C) receive domestic, hospital and industrial wastewaters and effluents containing the anticancer drug CP in ng/L levels. In general, the detected concentrations of CP measured in the influent and effluent were lower than those previously reported at a number of STPs in England (Booker et al., 2014). For this study, many of the samples were collected as composite samples over a 24 hr period and as such displayed a narrower range in concentration, with a lower variation compared to grab samples. The 24-hr composite samples did not show any significant loss of CP during STP treatment processes. The changes in concentration from the influent to the final effluent in this study were not statistically different when a

comparison was made between the treatment stages (influent, after primary treatment, post-UV). However, the influent composite samples (collected over 24 hr periods) do not represent the same wastewater that was sampled in the final effluent.

For many STPs serving urban catchments their morning and early evening raw influent flow rates (under typical low flow conditions) are higher than other periods of the day. It is plausible that the level of human pharmaceuticals present in raw influent would also be higher during this morning 'flush'. Therefore, to determine the fate of CP between the different processing stages and to attempt a mass balance over a relatively short time scale (<24 hr), then grab-sample data were used, these data relate to the same parcel of water passing through the respective STPs and are based on the HRTs for the sampling periods shown in Table 2.

Table 4 reports the output loads for STP-S as mean CP values (mg/d). The quantities of CP discharged into the environment are calculated by multiplying the average concentration by an average daily flow rate (range 32 - 36 ML/day), reported in Table 4. The total amount of CP discharged by STP-S with final effluent exceeds 18±8 g/year.

3.2. Mass balance for CP in STPs operating tertiary treatment

As described above grab samples were collected at carefully assessed time intervals (calculated from ancillary data shown in Table 2) to allow sampling of the same influent water as it progressed its way through the respective STP. Results from grab sampling for STP-S and STP-C are summarised in Figure 3a and 3b, respectively, with the box plot showing the range of concentrations (ng/L) of CP for each step sampled in the treatment process. The relative standard deviations (RSD%s) for CP detected in the sewage works by grab sampling were between 3 and 20% for STP-S

and between 11 and 13% for STP-C. Six measurements are given for STP-S (Figure 3a) and CP increases within the plant from 0.12 ng/L in the influent (RSD = 4%) to 0.18 ng/L post-UV treatment (RSD = 20%). Five measured values are provided for STP-C (Figure 3b) and again CP increases from being < MDL in the raw influent to 1.36ng/L being released post-UV treatment (RSD =13%). Results from grab sampling for STP-S and STP-C are presented as environmental loads (g/year) in Table 4. As CP passes through the STPs there is a notable increase in the concentrations and hence flux of the chemical in both treatment plants. Previous studies have shown that the elimination of CP in STPs is not fully removed from the wastewater during treatment processes (Buerge et al., 2006, Thomas et al., 2007, Martín et al., 2011, Llewellyn et al., 2011). The majority of studies reporting CP in STP wastewater show concentrations ranging from e.g. > MDL-43.8 ng/L with similar concentrations observed between the influent (median = 5.8 ng/L) and final effluent (median =6.3 ng/L), with comparable results for IF (Negreira et al., 2014, Zhang et al., 2013). However, several studies do show increases in the effluent concentration of CP, although this observation was not acknowledged, possibly due to sampling limitations, whereby there was a disconnect between the raw influent and the final effluent with different water parcels sampled (Buerge et al., 2006, Llewellyn et al., 2011). To our knowledge this study is the first study to examine concentrations of CP in the same parcel of wastewater as it passes through the various stages of treatment.

From Figure 3 there is a clear increase in CP concentrations moving from primary treatment to final post-UV effluents at both STP-S and STP-C. While the concentrations are not high at both plants relative to earlier studies this increase is statistically significant. The results here suggest that a proportion of CP is entering the STP in raw influent in a conjugated or bound form which is not detected using our

current analytical methodology (detailed above). As CP progresses through the treatment steps, then more of the CP is 'liberated' and is detectable. There is potential for CP reactivation from its subsequent conjugate during treatment leading to an increase in levels relative to the influent wastewaters (Kumar et al., 2012). Glucuronide and sulphate conjugates are phase II metabolites that leave the biologically active moiety of the parent drug intact, this phenomenon has been observed for other pharmaceuticals (Khan and Ongerth, 2004). For example, the rapid hydrolysis of paracetamol glucuronide has been observed in batch scale sewage investigation (Khan and Ongerth, 2004). The effect of this phenomenon is that studies using influent concentrations only are likely to underestimate the quantity of CP within the STP and its subsequent release into surface waters with final effluent. This also has the effect of confounding a mass balance approach. For example, the removal efficiency is effectively reversed, whereby CP is formed within the plant, rather than removed/eliminated. For example, taking the data as they are and assuming liberation or reactivation of CP from a bound or conjugated form (most notably after primary and then secondary treatment – see Figure 3) then there is no removal of the CP within the STP and a mass balance is not achieved with formation of CP within the STP (147±24% for STP-S and 1131±152% for STP-C).

CP was measured above the LOD in raw influent entering STP-S, but was not measured (below LOD) in influent entering STP-C, therefore the LOD was used at 0.12 ng/L to generate an annual input flux of 4.56 g/year when calculating the elimination efficiency. The HRT during the treatment at STP-S on the day of sampling was 7.14 hr and the HRT for STP-C on the day of sampling was 25.6 hr (almost 4 fold longer than STP-S). The difference in CP concentrations between STP-

S and STP-C may be related to the drug consumption and hospital proximity rather than their elimination efficiencies. (Llewellyn et al., 2011).

The HRT of wastewater in primary treatment was 2.69 hr for STP-S and 3.43 hr for STP-C. In both cases primary treatment resulted in extensive reactivation of CP. This is illustrated in Figure 3 and demonstrated in Figure 4 especially apparent STP-C where no CP was detectable in the influent, but after a HRT of 3.43 hours in the primary settling tanks CP was detectable with an average concentration of 0.27±0.03 ng/L (10.24±1.15 g/year is detectable). Both STP-S and STP-C utilise similar processes for their primary treatment. For CP, removal is not apparent during primary treatment with evidence suggesting that CP is 'reactivated' during wastewater retention in the settling tanks. A similar result has been demonstrated for oestrogens where an absence of removal was also noted during primary treatment (Gabet-Giraud et al., 2010). During primary treatment flocculation processes enhance the removal of suspended solids, thus allowing the settling and hence removal of particle-bound chemicals from the water body. Carbamazepine and ibuprofen with log K_{ow} in the range of 2.4-5.3 were not affected by coagulation/flocculation and settling of particulate matter and hence no removal was seen for these compounds (Carballa et al., 2005). A similar process is expected for CP which possesses a much lower K_{ow} (log K_{ow} = 0.63) with a high aqueous solubility and hence is unlikely to be lost through particle settling during primary treatment (Booker et al., 2014). Literature on the removal of pharmaceuticals by sedimentation processes is scarce, and data are often related to more than one treatment method (activated sludge) (Khan and Ongerth, 2004, Kumar et al., 2012). Removal of CP to sludge during primary treatment may occur for its bound or conjugated form, but the evidence here demonstrates an increase in CP between raw influent and primary effluent, indicating

that CP is liberated or reactivated from a bound/conjugated form. Secondary treatment involves the process of introducing oxygen into primary treated sewage combined with organisms to develop a biological flocculation which reduces the organic content of the sewage. The removal of CP from the aqueous phase during treatment with AS was calculated. In both STP-S and STP-C AS was shown to be ineffective as an elimination process for this chemical. A 33.2% increase in mass was detected in STP-S, when CP was incubated in the aeration lanes for 1.2 hr. Similarly in STP-C during both secondary and tertiary treatment CP increased in mass by ~475% (Figure 4-b). Previously published studies on pharmaceutical fate during incubation with AS show varied results, with some compounds showing no sorption onto the sludge/particle matter (e.g. ibuprofen) (Carballa et al., 2005). Other compounds that are positively charged (i.e. in an ionised form at neutral pH) can show removal due to electrostatic attraction with negatively charged groups at the surface of the AS (e.g. diclofenac, (Jelic et al., 2011)). However CP and IF are not ionisable (Booker et al., 2014) and thus removal is unlikely via this route.

The removal during tertiary treatment was evaluated for STP-S, with surface sorption to the low particle content of this effluent considered negligible (Gabet-Giraud et al., 2010). Figure 4-a reveals that during tertiary treatment CP is not degraded/removed, but neither is it reactivated from a conjugated form of CP (i.e. concentrations do not increase). Of the different treatment stages in STP-S, the tertiary treatment has the longest HRT (3.20 hr). In both STP-S and STP-C there is a final treatment process (UV) in operation. The results here demonstrate that this is the most effective stage of treatment for removing CP; however 80.8% of the CP still persists between the pre-UV and post-UV samples in STP-S. For STP-C, the UV treatment doesn't appear to have an effect on CP concentrations (see Figure 4b). Exposure of aqueous CP to UV

light is not considered to be a significant loss pathway, as the persistence of CP and IF was confirmed when exposed to UV treatment through irradiated lake water experiments (with a proposed half-life of 44 days for CP) (Buerge et al., 2006). Advanced oxidation processes (e.g. UV in the presence of H_2O_2) can provide a significant degradation route for CP (Franquet-Greil et al, 2015 – SETAC Barcelona, 2015 poster).

Elimination of CP during waste water treatment processes is very poor with final treated effluent concentrations of CP for both STPs frequently greater than the concentrations in raw influent. Poor STP elimination of this drug has been reported by others (Buerge et al., 2006, Thomas et al., 2007, Martín et al., 2011, Llewellyn et al., 2011). Effluent concentrations greater than those observed for the influent may be explained by the presence of a CP metabolite (possibly a conjugate) that is subsequently transformed by biotic and/or abiotic processes, particularly during primary treatment into the parent chemical. Similar to oestrogens, CP has the potential to occur as a glucuronide conjugate in sewage influent that could presumably, be cleaved in sewage, thus increasing its environmental concentration (Kumar et al., 2012). Because these conjugates were not included in the analysis, no firm conclusion can be made about their biotransformation. However it is apparent that CP (a compound with low adsorption coefficient) remains in the aqueous phase which favours their mobility through the STP and into receiving waters (Kosjek and Heath, 2011).

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Table 1: Flow characteristics of STP-S and STP-C

	STP-S	STP-C
Dry weather flow	30 ML/day	190 ML/day
Max Flow to full treatment	87 ML/day	234 ML/day
Average Flow	35 ML/day	101 ML/day

Table 2: Flow characteristics and HRTs (Hydraulic retention time) for STP-S and STP-C during grab sampling campaigns

	STP-S	STP-C
Flow to full treatment (ML/day)	72.5	104
Primary tank volume each (m3)	2708	2124
No of tanks in use	3	7
Primary tank retention time (h)	2.69	3.43
Secondary Aeration tank volume (m3)	3750	49000
Aeration tank retention time (h)	1.24	11.31
Tertiary Final tank volume each (m3)	2420	5897
No of tanks in use	4	8
Final tank retention time (h)	3.20	10.89
Retention time in plant	7.14	25.63

Table 3: Summary of 24-h composite samples and grab samples for CP at STP-S and STP-C, demonstrated as concentrations in ng/L. Standard deviated is shown where n > 2.

Treatment stage	STP-S (ng/L)	STP-C (ng/L)
Storm return	0.25±0.03	NA
Raw Influent	0.12 – 4.31±0.04	NF
Primary effluent	0.14±0.01 – 3.52±0.21	0.27±0.03
Aeration effluent	0.19±0.01	0.82
Pre-UV	0.22±0.02	1.28±0.14
Post-UV	0.18±0.04 – 2.36±0.01	1.36±0.18

NA – Not applicable
NF – Not found

Table 4: Calculated flux (mg/day) of CP at STP-S using 24-h composite sampling.

Standard deviated shown in brackets (n=2).

Date of sampling	Average wastewater daily flow ML/day	Mass load (mg/day) detected at each step			Mass in effluent (%)
		Raw Influent	After Primary	After tertiary i.e. Post-UV	
Mon 22/10/2012	32	56.11 (14.87)	69.42 (5.09)	32.19 (0.05)	57.37
Tue 23/10/2012	36	39.49 (1.58)	78.55	75.29 (1.96)	190.66
Wednesday 24/10/2012	35	38.43 (2.38)	43.89 (0.40)	70.72 (3.14)	184.09
Thursday 25/10/2012	34	36.35	29.78 (1.20)	34.63 (4.50)	95.28
Friday 26/10/2012	33	48.54	32.79 (0.30)	28.89 (2.78)	59.52
Saturday 27/10/2012	30	129.15 (1.15)	105.69 (6.45)	70.80 (0.17)	54.82
Sunday 28/10/2012	33	4.55	9.90 (1.87)	33.78 (3.06)	741.67
Average	33	50.38 (38.30)	52.86 (33.13)	49.47 (21.45)	98.20

Mass flux (mg/day) was calculated according to Eqn 1

Table 5: Calculated mass load (g/year) of CP at STP-S and STP-C during grab sampling campaign "Parcel of water". Standard deviated shown in brackets (n=3).

Date and sampling location	Daily flow rate (ML/day)	Mass load (g/year) detected at each step					
		Storm return	Influent	After Primary	After Secondary	Pre-UV	Post-UV
STP-S 8th October 2012	72.5	6.48 (0.86)	3.16 (0.13)	3.83 (0.38)	5.07 (0.16)	5.37 (0.49)	4.63 (0.94)
STP-C 18th to 19th November 2014	104	NA	NF	10.24 (1.15)	10.41*	48.42 (5.16)	51.57 (6.91)

* Sample was obtained at random (not collected at the correct time interval with the respect to the HRT and therefore represents a different water parcel)
NA – Not applicable
NF – Not found

Figure 1: STP ariel photograph for (a) STP-S, (b) STP-C

(a)

(b)

Figure 2: CP concentrations (ng/L) in the influent, primary effluent (after primary treatment) and post UV effluent wastewaters for 24-hr composite samples taken over a 7 day period.

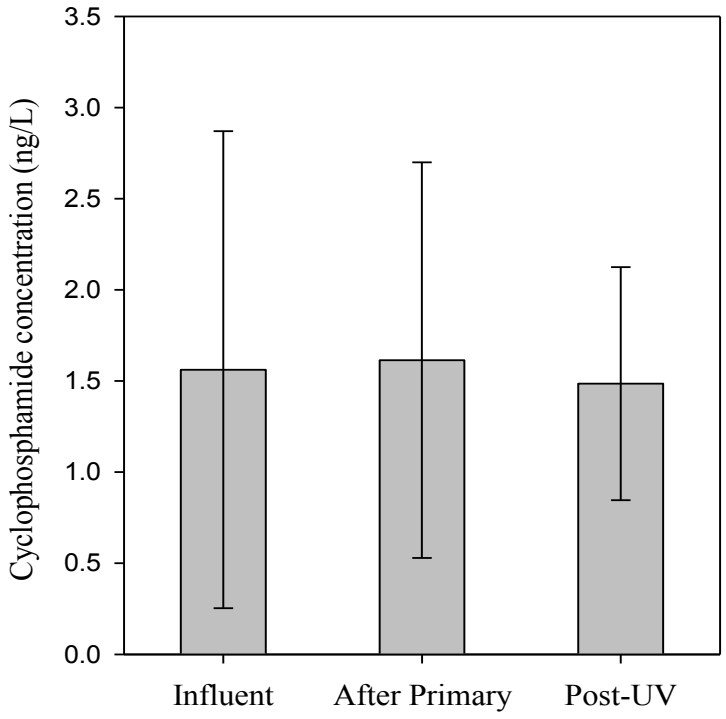
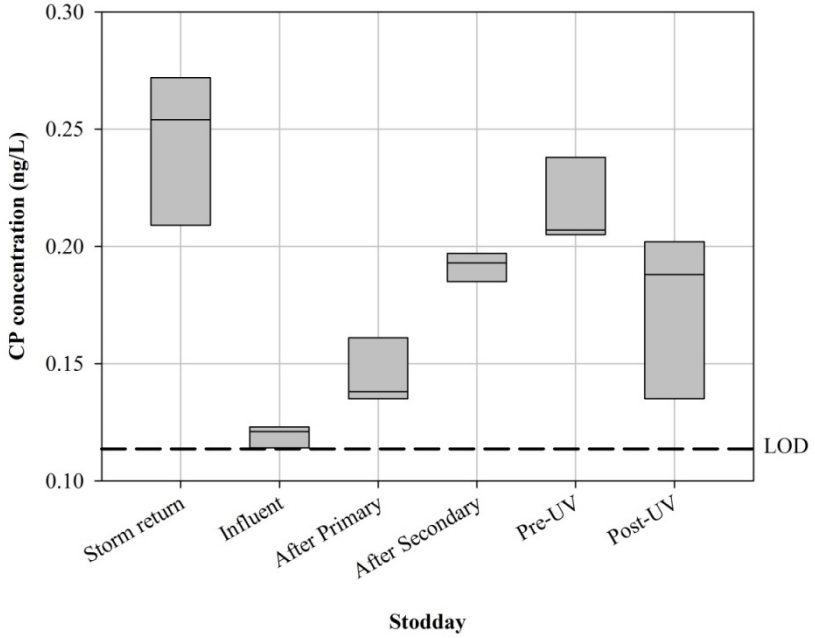


Figure 3: Box plot summary of CP concentrations (ng/L) in (a) STP-S (Stodday) and (b) STP-C (Clifton Marsh)



(b)

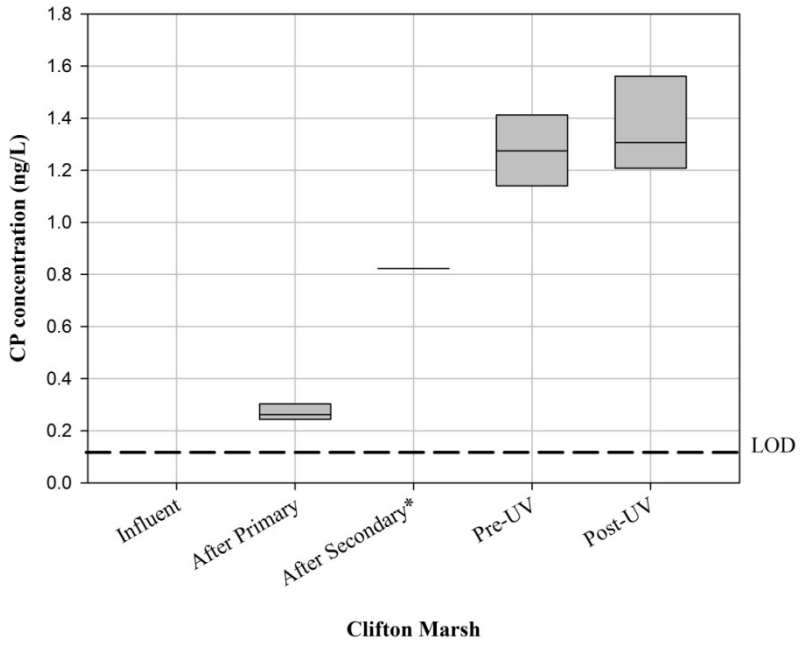
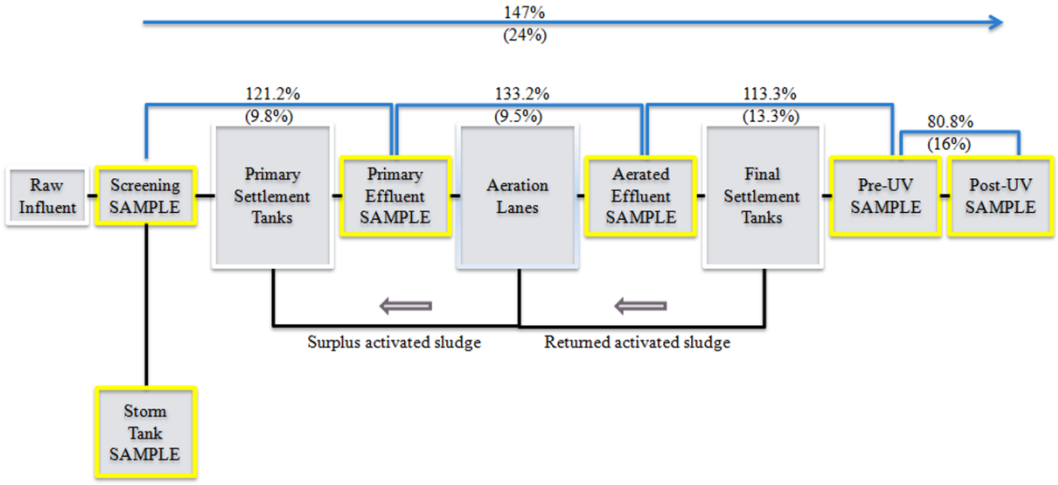
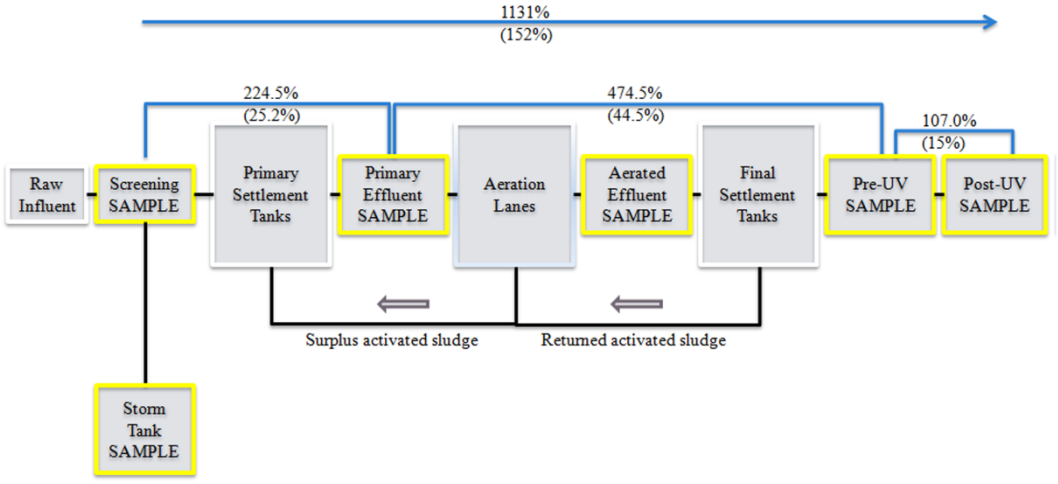


Figure 4: Schematic of (a) STP-S and (b) STP-C showing their treatment facilities and their calculated removal efficiencies for CP. Highlighted boxes (SAMPLE) show the sampling locations along the STPs

(a)



(b)



Paper IV

Modelling and measurement of the anticancer drugs, cyclophosphamide and ifosfamide, in the River Aire and Calder basin (UK).

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KEYWORDS

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Abstract

The regional consumption of anticancer drugs in the Aire catchment (NE England) and their subsequent fate in the aquatic environment was investigated using a catchment based water-quality model and accompanied by river water measurements. A shortlist of 10 priority drugs with population-based consumption data were used as input to a GIS-based water quality model (LF2000-WQX). Predicted environmental concentrations along the Aire and Calder rivers were generated with modelled concentrations compared to measured concentrations for two of these chemicals; CP and IF. The measured environmental concentrations (MECs) of CP ranged from 0.17 to 4.53 ng/L (average 1.14 ng/L) whereas IF showed lower concentrations ranging from <LOD to 1.82 ng/L (average 0.51 ng/L) and was not as frequently detected as CP. Comparison of modelled to measured river concentrations were generally good, although for the River Aire the MECs of CP were ~2.5-fold higher than PECs after the major conurbation of Leeds, ~65 km downstream from the first sampling point. Better agreement was observed on the River Calder, although the effect of specific STPs greatly elevated MECs above PECs for certain locations, yet a spike in concentration is observed at the lower end of the Calder that occurs in a reach that does not have a close upstream STP. Reasons for discrepancies between modelled and measured data are discussed. Risk quotients – the ratios of PECs to predicted no-effect concentrations (PNECs) derived largely from *Daphnia magna* toxicity assays were all <1 in this study, indicating that the risk of acute toxicity to sentinel aquatic invertebrates through exposure to common-use anticancer drugs is low.

1. Introduction

Cyclophosphamide (CP) and ifosfamide (IF) are two widely used alkylating agents belonging to the specialised class of anticancer drugs, the nitrogen mustard analogues (categorised as L01AA under the Anatomical Therapeutic Chemical (ATC) Classification system), used in chemotherapy as a treatment to a variety of cancers (Booker, Halsall et al. 2014).

In France a marked rise in the use of anticancer drugs has been demonstrated between 2004 and 2008, where CP consumption has increased by 8.5%, subsequently leading to higher environmental levels (Besse, Latour et al. 2012). The mean European consumption of CP is 10.4 µg/capita/day (Johnson, Oldenkamp et al. 2013), with France and the UK having approximate consumptions of 13.01 and 39.50 µg/capita/day, respectively. IF consumption is lower, with a UK consumption estimated to be 0.65 µg/capita/day whereas in France this is estimated to be 4.4 µg/capita/day in the UK (Besse, Latour et al. 2012; Booker, Halsall et al. 2014). Regional discrepancies in consumption of the anticancer drug fluctuate, with the River Aire catchment consuming only 2 µg/capita/day of CP and 2 µg/capita/day of IF (calculated from regional hospital data). Whilst the consumption of this specialised class of pharmaceuticals is much lower than for commonly prescribed or 'over the counter drugs', such as codeine (308.23 µg/capita/day in the UK) (Baker, Barron et al. 2014) their high toxicity gives reason for concern. Anticancer drugs have a high pharmacological potency and are designed to halt cell division and induce subtle genetic changes at low doses, making them increasingly targeted in environmental monitoring and risk assessment programmes (Johnson, Jürgens et al. 2008; Ferrando-Climent, Rodriguez-Mozaz et al. 2014).

The growing consumption of anticancer drugs suggests that concentrations in wastewater, final effluents and river water may increase, although this will be dependent on local/regional consumption, sewage treatment processes and river water flow and dilution (Booker, Halsall et al.). To date there are relatively few data on the occurrence of anticancer drugs such as CP and IF in surface waters and this remains a handicap for undertaking thorough risk assessments. Analytical improvements (e.g. MDLs ranging from ~10 ng/L in 1998 to < 1 ng/L in 2011) with liquid chromatography tandem mass spectrometry (LC-MS/MS) have led to a number of these chemicals being reported in hospital waste effluents, sewage treatment plant (STP) wastewaters and river waters in an increasing number of studies (Castiglioni, Bagnati et al. 2005; Buerge, Buser et al. 2006; Garcia-Ac, Segura et al. 2009; Yin, Shao et al. 2010; Llewellyn, Lloyd et al. 2011; Martín, Camacho-Muñoz et al. 2011).

Understanding the processes that determine the fate of CP and IF in the aquatic environment are crucial for predicting environmental concentrations at the whole catchment scale but rely on accurate hydrological data, pharmaceutical consumption data and knowledge of the chemical fate processes within STPs. Water quality and exposure models such as the GREAT-ER (Geography referenced Regional Exposure Assessment Tool for European Rivers) and LF2000-WQX (Low Flow 2000 – Water Quality Exposure) have been used to successfully to predict environmental concentrations (PEC) for a variety of organic contaminants in river systems (Boeije, Vanrolleghem et al. 1997; Feijtel, Boeije et al. 1997; Williams, Johnson et al. 2003; Williams, Churchley et al. 2012). However, few studies 'ground truth' modelled PEC with a coordinated measurement campaign to verify modelled results. This is important as variations in chemical emissions both spatially and temporally, as well as varying hydrology and un-quantified chemical fate processes may result in

discrepancies between predicted and observed behaviour for the contaminant in question (Sabaliunas, Webb et al. 2003). Authenticity of models may be accomplished via the use of measured environmental data.

The aim of this study was to use the catchment chemical fate model (LF2000 –WQX) to predict environmental river water concentrations for a selection of common-use anticancer drugs and compare these predictions to measured environmental concentrations for two of these chemicals. Two rivers, the Aire and Calder, were selected based on previous chemical fate studies (Schowanek and Webb 2002) with both arising in sparsely populated uplands with lower reaches passing through heavily urbanized areas served by a variety of sewage treatment plants (STPs) that discharge treated effluent (the source of anticancer drugs) into the rivers.

2. Methods

2.1. Study area and sampling

In this study, fourteen sampling locations were selected along the River Aire (catchment area approximately 282 km^2), which measures 115 km in length and is a major river in Yorkshire (UK). The Aire rises at Malham Tarn and flows through West Yorkshire to Gargave (population 1,764), Skipton (population 14,313), Keighley (population 89,870), Bingley (population 19,884), Shipley (population 28,162), Leeds (population 757,700), Swillington (population 3,530) and Woodlesford (population 21,010). Castleford (population 39,192) is the confluence of the Aire and Calder.

Eleven sampling locations were selected along the River Calder (catchment area approximately 341km^2), which measures 72 km in length and is a major tributary

river that rises on the eastern slopes of the Pennines and flows through the large urban and rural borough before joining the River Aire at Castleford. The Calder flows through villages, as well as the large towns of Brighouse (population 32,360), Mirfield (population 18,621), Dewsbury (population 62,945) and Wakefield (population 76,886). Sampling locations are shown for both the River Aire and Calder in Figure 1, including the STPs located along the catchment.

Two sampling campaigns were conducted with river water collected by grab sampling on consecutive days in April 2013 (30.04.2013 to 01.05.2013) and July 2013 (24.07.2013 to 25.07.2013). Sampling locations targeted upstream and downstream of the major STPs and near the confluence of the River Aire and Calder, the only exception is sampling point A7 (STP-1) which is a sampled STP effluent discharge stream on the River Aire. Duplicate samples were collected in 2.5L amber glass bottles (pre-washed and methanol rinsed), on return to the lab all samples (500mL) were filtered with GF/F filter paper (Whatman, UK), spiked with 5µL of deuterated-cyclophosphamide (d4-CP) internal standard (IS) for quantification purposes and stored at 4°C.

Precipitation in the week before sampling in April 2013 was low at 10.6 mm/week and 11.3 mm/week in the Aire and Calder catchments, respectively. There was minimal precipitation on the days of sampling (30/04/2013 to 01/05/2013). Precipitation in the week before sampling in July 2013 was higher at 23.2 mm/week and 27.7 mm/week in the Aire and Calder catchments, respectively. Precipitation was low on the days of sampling 24-25 July 2013 with rainfall 1.0 mm/day and 5.1 mm/day, respectively. Data were averaged from five locations in the catchment (www.worldweatheronline.com).

Gauged flow data are available for the River Aire at Lemonroyd (NGR SE381282) and for the River Calder at Methley (NGR SE408257). The daily flow values for the sampling days were obtained from the National River Flow Archive (CEH) http://www.ceh.ac.uk/data/nrfa/. The flow data were close to the Q95 low flow percentiles for the sites based on a 28 year daily flow record. Thus, any concentrations reported would represent the highest expected exposures that wildlife would experience in these rivers. Hydrological data are provided in Table 1.

2.2. Sample preparation and chemical analysis

Chemical standards of cyclophosphamide monohydrate (2-[Bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide) and ifosfamide (N,3-Bis(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine-2-oxide) were obtained from Sigma Aldrich (Gillingham, Dorset, UK). Custom-synthesized internal standard (IS) of deuterated-cyclophosphamide (d4-cyclophosphamide or d4-CP) was purchased from Qventas Laboratories (Branford, CT, USA), with HPLC-grade solvents purchased from Rathburn chemicals (Walkerburn, Scotland). Calibration curves were performed at seven levels, ranging from 0.14 to 6.63 ng/L for IF and from 0.22 to 10.33 ng/L for CP, in mobile phase (95% water, 5% methanol, 0.1% formic acid), each calibrate contained ~5µL IS d4-CP, resulting in a concentration of 15 ng/L.

Extraction and analysis of the samples followed a previously described method (Llewellyn, Lloyd et al. 2011), where 500mL of filtered (GFF filters, Whatman, UK) sample was spiked with 5 µL of d4-CP IS for quantification purposes and extracted using Strata X 500mg cartridges (Phenomenex, Macclesfield; UK). After the pre-concentration step cartridges were dried and eluted with 10 mL of HPLC grade ethyl

acetate. The elute was then loaded onto 200 mg Florisil (Biotage, Uppsala; Sweden) and eluted with 10% methanol in ethyl acetate, concentrated under N_2 and reconstituted in 500 µL of mobile phase.

Analysis was performed with liquid chromatography coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific, Hemel Hemsptead; UK) interfaced with an ion max electrospray ionisation (ESI) and operated with Xcalibur software ™ (V.2.0.7.). Analyte separation (10 µL inj. volume) was performed on a µPLC Hypersil GOLD C_{18} column (50 x 2.1 mm 1.9 µm) using a H_2O:MeOH mobile phase gradient (Llewellyn, Lloyd et al. 2011). Chromatographic peaks were integrated using the ICIS algorithm of Xcalibur™ which was also used to generate linear calibration curves using a 1/X weighting. Analyte peak signal to noise (S/N-RMS) ratios were obtained with the manual noise region option in Xcalibur™.

2.3. Hospital consumption

Table 2 presents consumption data obtained for hospitals within the River Aire and Calder catchment (West Yorkshire, Northern England). For this survey two of the National Health Service (NHS) trusts within the region were included to calculate their annual (kg/year) and per capita consumption (µg/cap/d). The Airedale NHS Foundation Trust contains collective information from Airedale general hospital, Skipton general hospital and Castleberg hospital, serving a population of 238,503. The Leeds teaching hospitals NHS trust contains collective information from Leeds general infirmary, St James' University hospital, Chapel Allerton hospital, Leeds Children's hospital, Seacroft hospital and Wharfedale hospital, serving a population of 2,600,000.

2.4. Geographic based exposure modelling

Water quality modelling (Johnson, Jürgens et al. 2008) was conducted using the GIS-based water quality model, LF2000-WQX. This can predict river water concentrations of 'down the drain' chemicals throughout the river network of England and Wales using human population data and long-term meteorological records on rainfall and runoff (Williams, Keller et al. 2009). The model has been used previously to successfully model steroid oestrogens and has been found to give acceptable agreement with measurements (Jobling, Williams et al. 2006; Williams, Churchley et al. 2012). Concentrations of 10 anticancer drugs were predicted for the Aire and Calder catchments using the local consumption data described above as the starting point. The model was run using the STP per capita load (after biodegradation) as the output value for each individual chemical (Table 2) (sewage effluent value CP = 0.5 µg/cap/day) with the assumption of no in-stream degradation.

2.5. Risk assessment

Finally, using toxicity assay data for key aquatic invertebrates then the low-flow model was also used to calculate risk quotients for two of the anticancer drugs along the Aire and Calder river basin (Feijtel, Boeije et al. 1997). The risk quotient (RQ) was performed to express the risk of the anticancer drugs to the environment. It is calculated as a quotient of the predicted environmental concentration (PEC) derived from the LF2000-WQX model for imatinib (L01XE01), hydroxycarbamide (L01XX05), methotrexate (L01BA01), capecitabine (L01BC06) and fluorouracil (L01BC02) with predicted no effect concentrations (PNECs) gleaned from toxicity assays. RQ for ifosfamide (L01AA06) and cyclophosphamide (L01AA01) are

calculated as a quotient of the measured environmental concentrations (MEC) in the River Aire and Calder with results of the PNECs. RQ were calculated according to:

$$RQ = \frac{PEC}{PNEC} = \frac{PEC}{EC_{50/f}} \quad (1)$$

PNEC can also be estimated as the quotient of the toxicological relevant concentration (EC_{50}) and a modifying or safety factor (f = 1000). For this purpose, the EC_{50} for *Daphnia* associated to the anticancer drugs was used for RQ calculations, where values are shown in Table 3. For data interpretation, the maximum probable risk for ecological effects from contaminated water was followed (Franquet-Griell, Gómez-Canela et al. 2015):

RQ < 1.0 indicates no significant risk;

1.0 ≤ RQ < 10 indicates a small potential for adverse effects;

10 ≤ RQ < 100 indicates significant potential for adverse effects;

RQ ≥ 100 indicates that potential adverse effects should be expected.

3. Results and Discussion

3.1. Priority contaminants derived from regional hospital consumption data

Table 2 reports the PECs of the ten common-use anticancer drugs in STP effluents and river water based on hospital usage data (from NE England), populations they serve, mean human excretion rate and potential for drug (bio) degradation during sewage treatment processes. Full details of the prioritisation methodology are described by Booker et al., 2014. Regional factors, such as variation in consumption levels, excretion rates, STP removal rates, size of the receiving water body and limits

of detection in analytical procedures can account for differences in the values and make predictions of the anticancer drugs difficult. The predicted effluent load (μg/cap/d) was used as a parameter for calculating the distributions of concentrations for the anticancer drugs using the LF2000-WQX model and mean PECs for the River Calder and Aire at the 95th percentile are presented. Local factors, such as variation in consumption, STP processes, dilution to the receiving river, analytical method and MDLs can all account for differences for MECs and make predictions of the anticancer drugs difficult (Toolaram, Kümmerer et al. 2014).

Based on this assessment anticancer drugs have been prioritised exclusively for this river catchment. Imatinib (L01XE01) was the most consumed L01 drug and also the compound to have the highest PEC in the effluent (PEC_{eff}) and surface waters (PEC_{river}). Clinical studies show marginal elimination by urinary excretion (9%) and insignificant biodegradation (<1%), yet still resulting in a high PEC_{eff} (164.2 ng/L) and PEC_{river} (16.4 ng/L). Imatinib has been selected by other prioritisation methods (Besse, Latour et al. 2012; Booker, Halsall et al. 2014; Franquet-Griell, Gómez-Canela et al. 2015), however predicted to be present in effluent at much lower concentrations. The high PEC_{eff} is due to a significantly higher consumption (393 kg/year) compared with the lower consumption found in the NE England (20.4 kg/year) (Booker, Halsall et al. 2014). Imatinib shows that a higher proportion of the parent drug will be released unmetabolised in faeces (20%) and sorption to sludge will be a removal factor in STPs that hasn't been identified, with possibility of occurrence in soil, following dispersion of sewage sludge to farmland (Booker, Halsall et al. 2014).

The second highest calculated PEC_{eff} is azacitidine (L01BC07), a compound that has a significantly lower consumption than imatinib (15.5 kg/year) but has a high urinary excretion rate (68% of the parent chemical) and negligible biodegradation (<1%). This compound was not featured in a prioritisation listing (focused on NW England) due to its low consumption (0.07 kg/year) (Booker, Halsall et al. 2014), highlighting the importance of using regional consumption data when studying a particular catchment. Another anticancer agent, capecitabine (L01BC06), a pro-drug that is metabolised to 5-fluorouracil (5-FU) (L01BC02) would simply add to the load of 5-FU present in STPs and hence the average PECs for 5-FU presented in Table 2 is likely to be underestimated

Other compounds included in this study are nilotinib (L01XE08), hydroxycarbamide (L01XX05), methotrexate (L01BA01), sunitinib (L01XE04), ifosfamide (L01AA06) and cyclophosphamide (L01AA01). Cyclophosphamide is presented with the lowest PEC_{eff} and PEC_{river} values in Table 2, yet this compound has been previously identified in STP effluents and receiving waters at 5.55±4.84 ng/L and 1.36±1.49 ng/L, respectively (Booker, Halsall et al.). Ifosfamide was not detected in this NW England river basin study, probably due to its low consumption (Booker, Halsall et al.).

3.2. Cyclophosphamide and ifosfamide in River Aire and Calder

CP and IF were detected in 90% and 56% of sampling sites, respectively. Figure 2 presents the progression of CP along the River Aire (Figure 2-a) and Calder (Figure 2-b). IF was detected near the river source (average 0.14±0.05 ng/L) at A1 in the River Aire in April 2013 and this may be attributed to point runoff associated with a household or community septic tanks as a STP is not present in this area.

For CP (Figure 2-a) the highest concentration was measured at site A7 (STP-1) (average 4.53 ng/L) along the River Aire and C1 (average 1.74 ng/L) along the River Calder. CP concentrations ranged from 0.17 to 4.53 ng/L (average 1.14 ng/L) for the whole study catchment. The River Aire and Calder CP levels were comparable to those reported in a NW England river catchment study (average 1.36 ng/L) (Booker, Halsall et al.). Higher concentrations of CP were observed in the River Aire in July 2013 (average 2.0 ng/L) than for April 2013 (average 1.2 ng/L) despite higher river flows that may have had a diluting effect on river concentrations (Table 1). The higher concentrations of CP in the River Aire for July stem from sampling point A7 (STP-1) where a concentration of 6.1 ng/L was measured close to an STP effluent discharge point in the city of Leeds (versus 3.0 ng/L in April 2013). From A7 downstream to A14 concentrations of CP are higher compared to previous measurements conducted in April 2013.

For IF (Figure 2-b) the highest concentration was measured at site A14 (average 1.82 ng/L) along the River Aire and C8 (average 0.17 ng/L) along the River Calder. MECs for IF range from <LOD to 1.82 ng/L (average 0.51 ng/L) for this catchment study. The River Aire and Calder IF levels were higher than those reported in NW England river catchment study, where IF was <LOD (Booker, Halsall et al.) and this likely reflects the higher consumption of IF at the major hospital trusts in the Aire catchment (2 μg/cap/day versus 0.65 μg/cap/day in NW England (Booker, Halsall et al. 2014)). Higher concentrations of IF are seen in the River Aire in July 2013 from A7 (average 1.16 ng/L) than for April 2013 (average 0.6 ng/L) despite having greater dilution (Table 1). Similarly to CP, the high MECs for IF start from sampling point A7 (STP-1) where 1.76 ng/L of IF was detected at an effluent discharge point in Leeds (versus 0.60 ng/L in April 2013) and IF concentrations remain higher at

downstream sampling points in the River Aire. It is thought that this higher effluent load from Leeds STP could be attributed to an increased daily consumption (possible due to an outpatient clinic administering CP and IF).

In this study, STPs located in more populated areas (Leeds to Castleford), are likely to be the most important sources of CP, with the highest measured concentrations observed between A7 and A14 on the River Aire and at C6 (Brighouse) on the River Calder. The highest MECs for IF were between A13 to A14 (Beal to Chapel Haddlesey) on the River Aire and but with much lower levels measured in the River Calder.

3.3. Predicted anticancer concentrations in the Aire and Calder using LF2000-WQX

Predicted imatinib (L01XE01), azacitidine (L01BC07), nilotinib (L01XE08), hydroxycarbamide (L01XX05), methotrexate (L01BA01), capecitabine (L01BC06), 5-fluorouracil (L01BC02), sunitinib (L01XE04), ifosfamide (L01AA06) and cyclophosphamide (L01AA01) concentrations in the River Aire and Calder were generated using LF2000-WQX assuming a predicted per capita STP load after excretion and (bio) degradation (as listed in Table 2). PECs and MECs for both CP and IF are shown in Figures 1 and 2 along with the mean flow rate calculated from the LF2000-WQX model.

For the River Aire (Figure 1-a) PECs for CP are much lower (by approximately 2.5 fold) than the values measured in both April and July 2013. In addition, the measured concentrations appear to be strongly influenced by inputs from STPs, where a peak in concentrations was observed for A7 (STP-1), 65km downstream of Airton. The large

increase in river flow (40 m^3/sec) some 75 km downstream from Airton represents the confluence of the River Calder and River Aire. The modelled data are broadly in agreement with the measured data here, with concentrations remaining at ~1 ng/L for the remainder of the river length despite the dilution effect of the two rivers merging. The increase in concentrations, both predicted and measured some 40 km downstream of Airton (the first upstream sampling site) represents an increase of CP due to discharge from a large STP (>150,000 population) at Esholt (A5). The increase in concentrations representing the STP effluents at A5 are not present in the IF measured data, and the only STP that influences the river water concentrations is A7 (STP-1).

The River Calder (Figure 2-b) PECs appear to agree with the average MECs, but do not capture the localised increase in concentrations associated with inputs from the major STPs. For example, 10 km downstream from Widdop a concentration peak is observed at Halifax due to a STP serving a population between 100,000 to 150,000. Again at 25km another increase is observed due to a large STP (>150,000 population) at Brighouse with a further increase in concentrations possibly associated with discharges from the Wakefield STP which is not captured by the model.

Modelling for the eight other priority anticancer drugs was carried out for the River Aire and Calder using LF2000-WQX and the average 95th percentile data (ng/L) are reported in Table 2. The PEC_{river} are all approximately 2 fold greater than the values derived by the initial prioritisation method that assumed a 10-fold dilution from STP effluent to receiving waters (Table 2) (Booker, Halsall et al. 2014) . Both CP and IF have shown that the modelled data are a good representation of the environmental levels, if not a little underestimated and so we can expect the other anticancer agents listed to have concentrations similar to these predictions for the River Aire catchment.

The predictions could benefit from more accurate (bio) degradation estimates during sewage treatment processes.

3.4. Risk assessment

The risk assessment of anticancer drugs in the River Aire/Calder catchment was performed based on the 95th percentile mean PEC_{river} values generated using LF2000-WQX and the EC_{50} values obtained from the literature presented in Table 3 (Zounkova, Odraska et al. 2007; Zounkova, Kovalova et al. 2010; Brezovšek, Eleršek et al. 2014; Franquet-Griell, Gómez-Canela et al. 2015; Lutterbeck, Kern et al. 2015). Ecotoxicity data of these drugs are scarce, although short term toxicity in aquatic organisms, usually *Daphnia magna* was available (Franquet-Griell, Gómez-Canela et al. 2015). The toxicity endpoints for these drugs was found to be highly variable with the EC_{50} values ranging from 0.13 (5-fluorouracil) to 1795 mg/L (ifosfamide). Nonetheless, the RQ values for these drugs turned out to be <<1.0 indicating a very low risk with regards to acute toxicity effects to aquatic invertebrates. Imatinib was the drug which provided the highest RQ, calculated using the LC50 value of 2.3 mg/L for *P. Subcapitata* toxicity test resulting in the highest RQ of 0.027 in the River Aire at approximately 70-75 km downstream from Airton at site A7 (STP-1) and A8 (downstream of Leeds STP). A predictive study in NE Spain (Catalonia) found hydroxycarbamide to have the highest RQ (3.21E-04) for the L01 drugs, calculated with an EC_{50} value >100 mg/L (Franquet-Griell, Gómez-Canela et al. 2015).

Anticancer drugs are often administered as combinational chemotherapy where a mixture of agents are used in a standardised regime and they can therefore be expected to be released in mixtures in wastewaters (Booker, Halsall et al. 2014; Parrella, Kundi et al. 2014). To determine the cumulative effects the ∑RQ values of

all compounds showed low risk in the River Aire (max 0.048) and the River Calder (max 0.037). These values are higher than those predicted in Spain (RQ 0.014), which was calculated using a worst case scenario and included a list of 25 chemicals, some from a different ATC classification (Franquet-Griell, Gómez-Canela et al. 2015). A study investigated the synergistic effect of long term exposure to 5-fluorouracil, cisplatin, etoposide and imatinib by testing binary mixtures in aquatic organisms (e.g. *D. magna*) and found that mixtures of the anticancer agents gave the same effect as exposure to a single drug , but at much lower concentrations (Parrella, Lavorgna et al. 2014). There is a lack of literature regarding the toxicity for complex mixtures of anticancer drugs in the environment. The low concentrations and their ability to interact with DNA makes these substances hard to define a 'safe level' and more sensitive toxicity assays addressing a range of chronic responses should be performed including effects arising due to exposures to mixtures (Parrella, Lavorgna et al. 2014; Franquet-Griell, Gómez-Canela et al. 2015).

Conclusions

This study presented the occurrence of cyclophosphamide and ifosfamide in a river network highly affected by urban and industrial pressures. Cyclophosphamide and ifosfamide were detected along the River Aire (UK) at high concentrations, reaching 6.1 ng/L for CP and 2.2 ng/L for IF. The highest concentration of these contaminants was observed near highly populated areas and wastewater discharges which were identified as an important source of these contaminants. Comparison of modelled to measured river concentrations was for most parts, reasonably predicted by LF2000-WQX. Underestimations occurred for CP in the River Aire after the major conurbation of Leeds, with measured concentrations elevating for the remainder of

the river length despite the dilution effect of the two rivers merging. Overall, the use of consumption data is good at predicting river water concentrations for the dissolved chemicals, like CP and IF and based on these predictions it should do a reasonable job for the ten priority agents in the River Aire and Calder. However, for more reactive drugs which show sufficient loss in STPs then LF2000-WQX may not perform as well. Where available, measured effluent data should be used to check and refine, if necessary, exposure assessments for such chemicals. A risk assessment was performed based on water concentration predictions and acute toxicity data. The cumulative effects of the sum of RQ values showed no risk of acute toxicity for aquatic invertebrates in either the River Aire or Calder. However, long term studies for these pollutants in the water environment are needed to define the environmental stress produced through the continuous exposure to anticancer drugs in these rivers.

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Figure 1: Map of sampling locations along the River Aire (A1 to A14) and along the River Calder (C1 to C11). STPs are illustrated; the triangle represents sewage works that serve a population of <50,000, a pentagon represents STPs with a population between 50,000 – 100,000, a circle represents STPs with a population between 100,000 – 150,000 and a square represents large sewage treatment plants serving a population over 150,000.

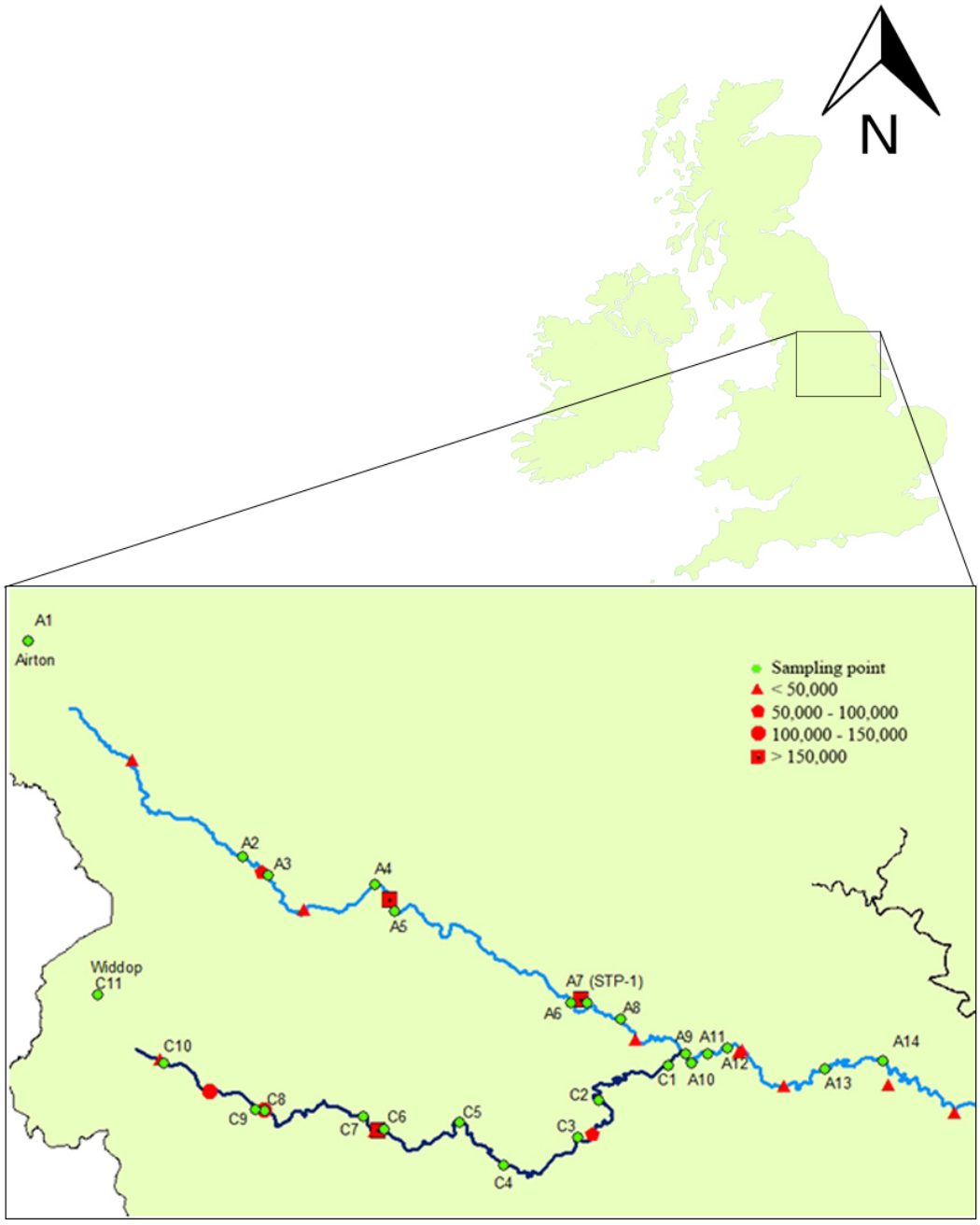
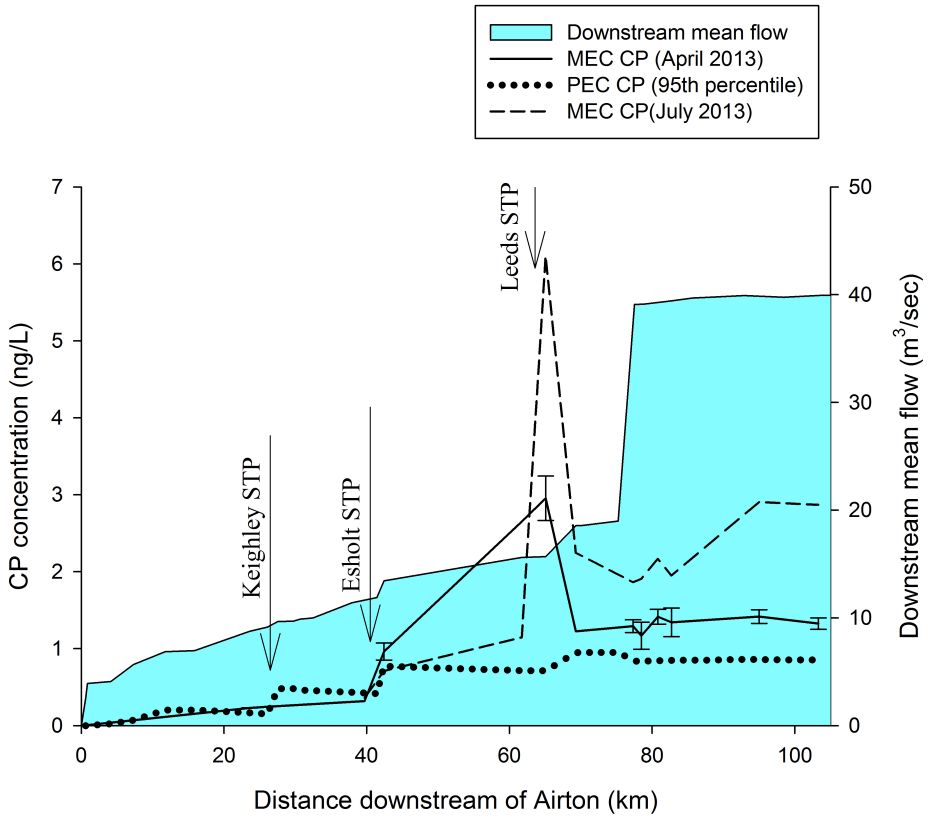


Figure 2: Measured environmental concentrations (MEC) of CP (April 2013 and July 2013) and predicted environmental concentrations (PEC) of CP along the River Aire, Yorkshire (NE England). PECs were calculated using regional consumption data with the assumption that there was no removal during sewage treatment (CP = 0.5 µg/cap/d). The solid dark line represents the MEC (April 2013), the dashed line represents the MEC (July 2013), the dotted line represents the 95th percentile PEC values and the shaded area represents the simulated downstream mean flow rate. (a) Downstream from Airton (River Aire) (Airton is the first sampling point near the upland source of the river) and (b) Downstream from Widdop (River Calder) (Widdop is the first sampling point close to the source of the river)

(a)



(b)

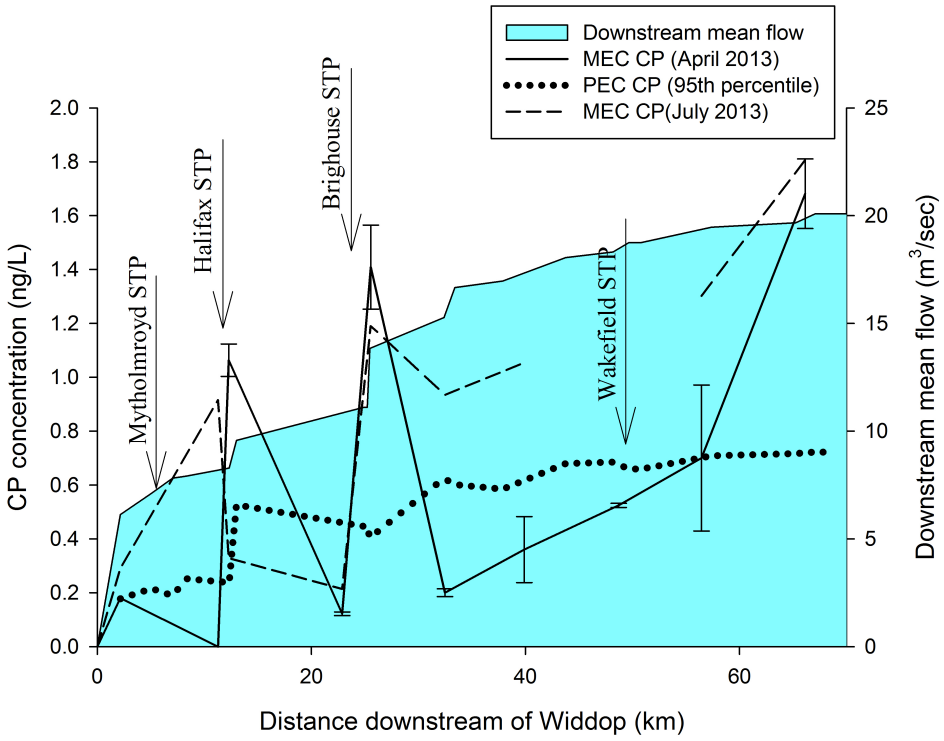

Downstream mean flow
MEC CP (April 2013)
PEC CP (95th percentile)
MEC CP(July 2013)
Mytholmroyd STP
Halifax STP
Brighouse STP
Wakefield STP
CP concentration (ng/L)
Downstream mean flow (m3/sec)
Distance downstream of Widdop (km)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
0
5
10
15
20
25
0
20
40
60

Figure 3: MEC of IF (April 2013 and July 2013) and PEC of IF downstream from Airton (River Aire) calculated using regional consumption data with the assumption that there was no removal during sewage treatment (IF = 0.5 µg/cap/d). The solid dark line represents the MEC (April 2013), the dashed line represents the MEC (July 2013), the dotted line represents the 95th percentile PEC values and the shaded area represents the simulated downstream mean flow rate.